Award Number: DAMD17-99-1-9552

TITLE: Opening of the Mitochondrial Permeability Transition Pore by Reactive Oxygen Species is a Basic Event in Neurodegeneration

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REPORT DATE: July 2003

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20031212 155

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	**2. REPORT DATE** July 2003	**3. REPORT TYPE AND DATES COVERED** Final Addendum (1 Jul 02 - 30 Jun 03)

4. TITLE AND SUBTITLE
Opening of the Mitochondrial Permeability Transition Pore by Reactive Oxygen Species is a Basic Event in Neurodegeneration

5. FUNDING NUMBERS
DAMD17-99-1-9552

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This addendum concludes the work on the neurotoxicity induced by Aβ which was an extension of our original work involving the administration of aluminum maltolate into rabbit brain. The intracisternal injection of Aβ(1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These Aβ effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that Aβ-induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau. In addition, signaling pathways activated by aluminum maltolate and Aβ injections into rabbit brain, are also activated by the injection of MPP^+ which is a neurotoxin that profoundly affects dopaminergic neurons. This MPP^+ model is of considerable value in understanding neuronal injury in Parkinson's disease. The most valuable information that has been obtained in our studies is that the endoplasmic reticulum, along with mitochondria, are immensely important in regulating cell death or survival via apoptosis mechanisms, following neurotoxic injury. We suggest that agents that prevent oxidative damage and endoplasmic reticulum stress will provide improved protection from aluminum, Aβ or MPP^+-induced neuronal injury.

14. SUBJECT TERMS
Neurotoxin

15. NUMBER OF PAGES
91

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	**18. SECURITY CLASSIFICATION OF THIS PAGE** Unclassified	**19. SECURITY CLASSIFICATION OF ABSTRACT** Unclassified	**20. LIMITATION OF ABSTRACT** Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION:

Our grant entitled "Opening of the mitochondrial permeability transition pore by reactive oxygen species is a basic event in neurodegeneration" was originally funded for the period July 1, 1999 through June 30, 2002. On July 27, 2001 we received supplemental funds of $179,228 to enable us to expand our research effort. We used some of these funds before June 30, 2002 and the results of our studies are given in the Final Report submitted on July 22, 2002. The remainder of the funds allowed us to continue our research for a few more months and the results are provided in this addendum to our final report.

BODY

OUTLINE OF EXPERIMENTS:

- Our work has continued with our original objective to study mechanisms of neuronal injury and death using the intracisternal administration of neurotoxins to New Zealand white rabbits. Our original work focused on aluminum maltolate as the neurotoxin administered. This toxic agent produced severe neurotoxic injury and we were able to provide some of the first results describing the endoplasmic reticulum as a target for this neurotoxin with subsequent cell death via an apoptotic mechanism. We also were able to modulate the toxicity by two agents, lithium or glial cell-line derived neurotrophic factor (GDNF). The mechanism by which these two agents appear to act is via protection against both mitochondrial and endoplasmic reticulum stress.

- After describing these novel pathways of *in vivo* neurotoxic action we extended our experiments to evaluate the intracisternal administration of the peptide Aß on the apoptosis pathways. Some of this work was described in our Final Report and is relevant to human neurodegeneration, since Aß forms the core of neuritic plaques that are seen in Alzheimer's disease. It is also known that Aß is neurotoxic, albeit not as toxic as the aluminum compounds. We have extended this work on Aß and the results are summarized in the present Report.

- We also realized the potential of our mechanistic studies to assess neuronal death in the substantia nigra, which is particularly relevant to Parkinson's disease. We chose to evaluate the neurotoxin MPP^+ which has been used as a model for Parkinson's disease, particularly in rats, mice and primates. Its use derived from the illegal use of the precursor drug, MPTP, several years ago and a drug which produced a distressingly high incidence of Parkinson's disease in human users. MPP^+ is the active metabolite of MPTP. Our exciting new results using this Parkinson's model system are also summarized in the present Report.

SUMMARY OF RESULTS:

Lithium inhibits Aβ-induced stress in the endoplasmic reticulum in rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation

This work was described in the Final Report but had not yet been published. Publication has now been accomplished in the **Journal of Neuroscience Research** and a reprint is attached as **Appendix I.**

Aβ(1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus.

We have demonstrated that the intracisternal injection of Aβ(1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These Aβ effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that Aβ-induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau.

This work has been submitted to **Molecular Brain Research** and a copy of the manuscript is attached as **Appendix II.**

Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain

Loss of neurons is a hallmark of neurodegenerative disorders and there is increasing evidence suggesting that apoptosis is a key mechanism by which neurons die in these diseases. Mitochondrial dysfunction has been implicated in this process of neuronal cell death but there is a growing body of evidence also suggesting an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondria or in concert with mitochondrial-initiated pathways. Studies in our laboratory have focused on neuronal injury resulting from the administration of aluminum maltolate, via the intracisternal route, to New Zealand white rabbits. Treatment of rabbits with aluminum maltolate induces both mitochondrial and endoplasmic reticulum stress. Agents such as lithium or GDNF have the ability to prevent aluminum-induced neuronal death by interfering with the mitochondrial and/or the endoplasmic reticulum-mediated apoptosis cascade. This animal model system, involving neurotoxicity induced by an aluminum compound, provides new information on the mechanisms of neurodegeneration and neuroprotection.

This work formed the basis of an oral presentation at the **Fifth Keele Meeting on Aluminum held in Stoke-on-Trent, United Kingdom, February 23-25, 2003.** A manuscript is now in press in **The Journal of Inorganic Biochemistry** and is attached as

Appendix III. The Abstract that was accepted for presentation at the Conference is attached as **Appendix IV**.

MPP$^+$ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of the ATF-6 and NF-κB signaling pathways.

Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie MPTP/MPP$^+$-induced neurotoxicity, used extensively as a model for Parkinson's disease. Signaling pathways utilized within other subcellular organelles have been less extensively explored. In the present study we have examined the hypothesis that MPP$^+$ targets other cell compartments, including the endoplasmic reticulum. Because rabbits possess more genetic similarities to primates than to rodents we have selected this animal model system for our MPP$^+$ neurotoxicity studies. MPP$^+$ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. We demonstrate that MPP$^+$ in a dose-dependent manner induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by the activation of ATF-6 and NF-κB, leads to activation of gadd 153 and caspase-3. These effects correlate with activation of the JNK kinase signaling pathway as well as phosphorylation of one of its substrates, c-jun. Our results demonstrate that MPP$^+$ directly targets the endoplasmic reticulum and is neurotoxic to rabbits by mechanisms that involve the activation of the endoplasmic reticulum stress response and JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

This work has been submitted for publication in the **Journal of Neuropathology and Experimental Neurology** and the paper is attached as **Appendix V**; an **Abstract (Appendix VI)** has been accepted for presentation at the **33rd Annual Meeting of the Society of Neuroscience** to be held in New Orleans, LA, November 2003.

Cellular mechanisms of neurodegeneration and Alzheimer's disease

A review of some aspects of our work was presented at the **Annual Meeting of the Association of Clinical Scientists** held in St Petersburg, FL, 7-11 May, 2003. The **Abstract** of this presentation is attached as **Appendix VII**.

Is amyloid β-peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions?

As stated in our Final Report, the editor of **Neurobiology of Aging** invited the P.I. to write a commentary on this topic. In this article we present our hypotheses concerning the interaction of Aβ and Al. This commentary is especially relevant in light of the recent publication on the enhanced neurotoxicity of Al in Aβ-producing transgenic mice (1). A preprint of our commentary was originally attached as an Appendix in our Final Report and we include a reprint attached to this Addendum as **Appendix VIII**.

KEY RESEARCH ACCOMPLISHMENTS:

Most of our Key Research Accomplishments from this project were included in our Final Report. Here we list additional Accomplishments for the past year.

- JNK and ERK kinase activation may mediate the Aβ-induced apoptosis; lithium and GDNF, although targeting these cellular signaling pathways in different ways, prevent the Aβ-induced caspase activation.
- MPP^+ directly targets the endoplasmic reticulum and is neurotoxic to rabbits by mechanisms that involve the activation of the endoplasmic reticulum stress response and JNK kinase.
- We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

REPORTABLE OUTCOMES:

In the Final Report we have listed thirteen full-length papers and ten abstracts that resulted from the project supported by the Department of Defense. Here we list four additional full-length papers either published or submitted, together with four Abstracts. One full-length paper which was included in our Final Report is also included in this Addendum since we now have the final reprint.

Papers:

Ghribi O, Herman MM, Savory J. Lithium inhibits Aβ-induced stress in endoplasmic reticulum of rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation. J Neurosci Res 71: 853-862, 2003 (Appendix I).

Ghribi O, Herman MM, Pramoonjago P, Savory J. Aβ(1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus. Molecular Brain Research, submitted (Appendix II).

Ghribi O, Herman MM, Savory J. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J Inorg Biochem, in press (Appendix III).

Ghribi O, Herman MM, Pramoonjago P, Savory J. MPP^+ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF-κB signaling pathways. J Neuropathol Exp Neurol, submitted (Appendix V).

Savory J, Ghribi O, Herman MM. Is amyloid β-peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions? (invited commentary) Neurobiol of Aging, 23: 1089-1092. 2002, (Appendix XIII). This paper was included in our Final Report as a preprint. The present Appendix XIII is a reprint.

Abstracts:

Ghribi O, Herman MM, Savory J. Aβ induces hyperphosphorylation of tau and apoptosis in rabbit brain, involving activation of the GSK-3β and JNK pathways. Society for Neuroscience 32th Annual Meeting, Orlando, FL, November 2-7, 2002, (Appendix IX).

Savory J, Ghribi O, Herman MM. Aluminum-induction of endoplasmic reticulum-specific apoptosis in experimental neurodegeneration and its reversal by lithium. Presented at the 5th Keele Meeting on Aluminum, Stoke-on-Trent, United Kingdom, 22nd-25th February, 2003, (Appendix IV).

Ghribi O, Herman MM, Pramoonjago P, Savory J. MPP^+ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF-κB signaling pathways. To be presented at the 33rd Annual Meeting of the Society of Neuroscience to be held in New Orleans, LA; November 2003, (Appendix VI).

Savory J, Ghribi O, Herman MM. Cellular mechanisms of neurodegeneration and Alzheimer's disease. Presented at the Annual Meeting of the Association of Clinical Scientist held in St Petersburg, FL, 7-11 May, 2003, (Appendix VII).

CONCLUSIONS:

An extensive discussion of our conclusions was included in our Final Report. To these conclusions we now add that the intracisternal injection of Aβ(1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These Aβ effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that Aβ-induced apoptosis and the phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau. In addition, signaling pathways activated by aluminum maltolate and Aβ injections into rabbit brain are also activated by the injection of MPP^+, which is a neurotoxin that profoundly affects dopaminergic neurons. This MPP^+ model is of considerable value in understanding neuronal injury in Parkinson's disease. The most valuable information that has been obtained in our studies is that following neurotoxic injury, the endoplasmic reticulum, along with mitochondria, are immensely important in regulating cell death or survival via apoptosis mechanisms. We suggest that agents that prevent oxidative damage and endoplasmic reticulum stress will provide improved protection from neuronal injury induced by aluminum, Aβ or MPP^+.

SO WHAT:

We now have a better understanding of how brain cells survive or die following the admistration of three important neurotoxins. Aluminum is highly toxic and is the most common metal in the environment. Aβ is deposited as neuritic plaques in Alzheimer's diease and its neurotoxicity is almost certainly relevant to understanding the pathogenesis of this devastating neurodegenerative disorder. MPP^+ is also highly neurotoxic and destroys dopaminergic neurons, a consequence of which are clinical symptoms of Parkinson's disease. We now know that both lithium and GDNF can ameliorate the toxicity induced by aluminum, Aβ, and probably by MPP^+, although the latter studies have not been carried out in our animal model system. We have a solid basis of evidence to support the use of these treatments in neurotoxin-exposed personnel.

REFERENCES CITED:

1. Pratico D, Uryu K, Sung S, Tang S, Trojanowski JQ, Lee VM. Aluminum modulates brain amyloidosis through oxidative stress in APP transgenic mice. FASEB J 2002;16:1138-40.

Note: An extensive list of references is included in Appendices I, II, III and V.

Personnel receiving salary support from this grant:

John Savory, Ph.D.

Othman Ghribi, Ph.D.

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APPENDIX I

Journal of Neuroscience Research 71:853–862 (2003)

Lithium Inhibits Aβ-Induced Stress in Endoplasmic Reticulum of Rabbit Hippocampus But Does Not Prevent Oxidative Damage and Tau Phosphorylation

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The goal of this study was to assess the in vivo effect of Aβ on apoptosis pathways involving the endoplasmic reticulum and mitochondria, and its relationship to the induction of tau phosphorylation and DNA oxidative damage. In rabbits treated intracisternally with aggregated Aβ(1–42), clear evidence of endoplasmic reticulum stress was observed by the activation of caspase-12 and cleavage of caspase-3 in the endoplasmic reticulum. Mitochondrial injury was evident from the release of cytochrome c into the cytosol and the induction of oxidized mitochondrial DNA. Tau phosphorylation and nuclear translocation of NF-κB and GSK-3β were also observed. Treatment with lithium, an inhibitor of GSK-3β, inhibited caspase activation but did not prevent mitochondrial DNA damage or tau hyperphosphorylation, suggesting that the translocation of GSK-3β may represent an upstream event that leads to caspase activation but is unrelated to tau hyperphosphorylation or mitochondrial DNA oxidative damage. We propose that treatment by lithium alone is not sufficient to protect against the multiple adverse effects of Aβ, and the use of agents that prevent oxidative DNA damage and tau hyperphosphorylation, together with lithium, may provide better protection from the neurotoxic effect of Aβ.
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Key words: caspase 3; caspase 12; cytochrome c; GSK-3β; NF-κB

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of extracellular neuritic plaques (NPs), the formation of neurofibrillary tangles (NFTs), and massive neuronal and synaptic loss. Aβ is the major component of NPs, with hyperphosphorylated tau being the primary constituent of NFTs; both of these aggregates are generally accepted as being key players in the pathogenesis of AD. Whether Aβ or tau actually represents the primary cause of neuronal loss in AD, and which of the two abnormal changes is generated first and is presumably the more important, remains a matter of debate. Transgenic mice with mutated amyloid precursor protein, the precursor of Aβ, can develop Aβ deposits (Duff et al., 1996; Citron et al., 1997; Gau et al., 2002) but NFTs have not been detected (Games et al., 1995; Gau et al., 2002). Also, transgenic mice over-expressing mutant tau do not systematically exhibit NP aggregates (for review see Mudher and Lovestone, 2002). There is convincing evidence that NPs and NFTs have some association, however, because transgenic mice over-expressing both mutant amyloid precursor protein and mutant tau exhibit marked neurofibrillary degeneration (Lewis et al., 2001). In accord with these findings is the report that injection of Aβ fibrils in P301L tau transgenic mice enhances the formation of NFTs (Gotz et al., 2001).

In vitro studies using cultured cells exposed to synthetic Aβ have demonstrated evidence of tau phosphorylation and neurodegeneration (Takashima et al., 1993, 1998; Alvarez et al., 1999). These latter results suggest that Aβ is the trigger of tau accumulation and neuronal death. It has been suggested that the mechanism by which Aβ induces tau hyperphosphorylation involves the activation of glycogen synthase kinase-3β (GSK-3β), an enzyme that plays a crucial role in signaling processes involved in psychiatric diseases such as bipolar disorder, and in neurodegenerative diseases such as Alzheimer's (for review see Grimes and Jope, 2001). Lithium, a medication for bipolar disorder, is a direct inhibitor of GSK-3β (Klein and Melton, 1996), and has been demonstrated to prevent hyper-

Contract grant sponsor: United States Department of the Army; Contract grant number: DAMD 17-99-1-9552; Contract grant sponsor: Alzheimer's and Related Diseases Research Award Fund, Virginia Center on Aging, Virginia Commonwealth University; Contract grant number: 02-3.

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Received 10 August 2002; Revised 3 October 2002; Accepted 7 October 2002

Published online 00 Month 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.10511

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phosphorylation of tau (Munoz-Montano et al., 1997) and the Aβ-induced degeneration of cultured neurons (Alvarez et al., 1999).

In addition to plaque deposition and tangle formation, there are other possible pathological hallmarks of AD, including oxidative stress (for review see Smith et al., 2000; Butterfield et al., 2001) or perturbed endoplasmic reticulum function (for review see Mattson et al., 2001; Mattson and Chan, 2001)]. It has been shown in mouse cortical neurons that Aβ triggers an endoplasmic reticulum-specific apoptosis mediated by active caspase-12, which may activate effector caspases such as caspase-3 (Nakagawa et al., 2000). Additionally, the transcription factor NF-κB seems to be linked to Aβ-evoked neuronal loss as well as to GSK-3β pathways (Hoeflich et al., 2000; Javelaud and Besancon, 2001; Tang et al., 2001).

We have used an in vivo system, involving the administration of aggregated Aβ(1–42) peptide into rabbit brain via the intracisternal route, which we have observed to induce hyperphosphorylation of tau, oxidative stress, and perturbation of the endoplasmic reticulum. We have further treated these animals with lithium, an inhibitor of GSK-3β (Bijur et al., 2000), which we have shown recently to inhibit aluminum-induced apoptosis in rabbit hippocampus (Ghribi et al., 2002b). We applied this treatment to determine if lithium inhibits Aβ-induced tau hyperphosphorylation and apoptosis.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies (mAbs) to caspase-3/CPP 32 and calnexin were obtained from Transduction Laboratories (Lexington, KY). Caspase-12 mAb was a gift from Dr. Junying Yuan (Harvard Medical School, Boston, MA); cytochrome c, cytochrome c oxidase subunit IV, NF-κB p50 and NF-κB p65, GSK-3β, p-Tyr^{216} GSK-3β, p-Ser^{9} GSK-3β, and histone H1 were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and the anti-human PHF tau /AT8 mAb was supplied by Innogenetics (Ghent, Belgium).

Animals, Treatment Protocol, Clinical Monitoring and Tissue Collection

Adult female New Zealand white rabbits (3–4 years weighing 4–5 kg) received either intracisternal injections of 100 μL normal saline (controls; *n* = 6), 100 μl of 400 μM Aβ(1–42) (*n* = 6; Aβ-treated group), or lithium and 100 μL of 400 μM Aβ(1–42) (lithium/Aβ-treated group; *n* = 6). Aβ(1–42), obtained from American Peptide Company (Sunnyvale, CA), was prepared by incubating freshly solubilized Aβ(1–42) in saline at 37°C for 3 days. The intracisternal injections were carried out over a period of 2 min under ketamine anesthesia as described previously (Ghribi et al., 2001a). Lithium carbonate (Sigma, St. Louis, MO) was administered orally by adjusting the drinking water to a concentration of 7 mM as described previously (Ghribi et al., 2002b). This concentration of lithium in the drinking water yields a serum concentration of 0.65 ± 0.35 mM (mean ± SEM), which is at the lower end of the therapeutic range used in humans (Moyer, 1999). Lithium was started 14 days before Aβ(1–42) administration and continued until animal sacrifice. All rabbits were sacrificed 7 days after the intracisternal administration, and at necropsy were perfused with Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY) at 37°C. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Animal Care and Use Committee.

Western Blot Analysis

Proteins from enriched fractions of nuclei, mitochondria, cytosol and microsomes were extracted as described previously (Ghribi et al., 2001b). Tissue from the entire hippocampus was homogenized gently using a Teflon homogenizer (Thomas, Philadelphia, PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 mg/ml of *N*-acetyl-Leu-Leu-Norleu-Al). The nuclear fraction was the first pellet (750 × *g*, 10 min) and the mitochondrial fraction was the second (8,000× *g*, 20 min). The supernatant was further centrifuged at 100,000× *g* for 60 min at 4°C to separate the cytosolic (soluble) from the microsomal (pelleted) fractions. The microsomal fraction is referred to as endoplasmic reticulum-enriched fraction, although vesicules and other membrane components are also included in this fraction, as confirmed previously by electron microscopy (Ghribi et al., 2002a) . Protein concentrations in all fractions were determined with the BCA protein assay reagent (Pierce, Rockford, IL).

Proteins (5 μg) from the nuclear, mitochondrial, cytosolic and endoplasmic reticulum-enriched fractions were separated by SDS-PAGE (10% gel), then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and incubated with mouse mAb to cytochrome c at a dilution of 1:500, caspase-3 (1:200), caspase-12 (1:10), NF-κB p65 and NF-κB p50 (1:250), GSK-3β, p-Tyr^{216} GSK-3β and p-Ser^{9} GSK-3β (1:500). Cytochrome c oxidase, calnexin, and histone H1 were used as markers for mitochondria, endoplasmic reticulum, and nuclei, respectively, and were diluted at 1:250. The blots were developed with enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA) and bands were analyzed by densitometry with Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry

Oxidative DNA damage. We used the OxyDNA assay kit (Calbiochem, San Diego, CA), which is based on the direct binding of a fluorescent probe to 8-oxoguanine moieties in the DNA of fixed tissue. Frozen sections (14 μm thick) at the mid-hippocampal level from control, Aβ(1–42)-treated and lithium/Aβ(1–42)-treated animals were air dried, fixed in ice-cold 4% paraformaldehyde in PBS for 15 min, washed briefly in TBS/Tween 20 (provided in the kit) and permeabilized in ice-cold 99% methanol for 30 min. After two washes, the sections were incubated with the provided blocking solution for

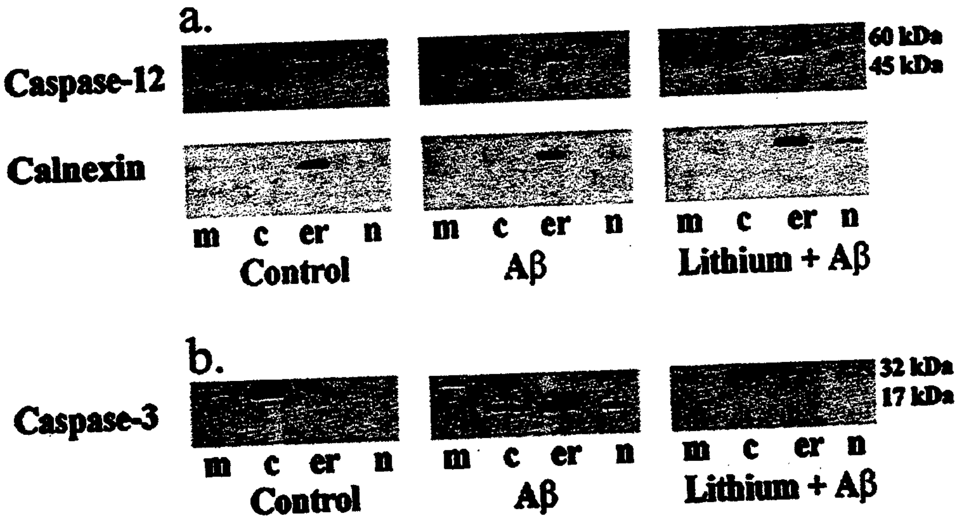

Fig. 1. Aβ induces endoplasmic reticulum stress. **A:** Caspase-12 predominantly resides in the endoplasmic reticulum fraction (er) as procaspase-12 (60 kDa) in control, Aβ-treated, and lithium/Aβ-treated animals. Aβ induces release of the active form of caspase-12 into the cytosol (c) and lithium prevents the Aβ-induced caspase-12 activation. Calnexin, used as an endoplasmic reticulum marker, stains predominantly the endoplasmic reticulum fractions (er). **B:** Procaspase-3 (32 kDa) is localized mainly in the cytosolic fractions (c) of control, Aβ-treated and lithium/Aβ-treated rabbits. Active caspase-3 (p17) is detected in the Aβ-treated animals only, and is localized in the endoplasmic reticulum (er) > cytosolic (c) > nuclear (n) > mitochondrial (m) fractions.

30 min at 37°C for 1 hr, washed twice in PBS, incubated overnight at 4°C with a 1:50 dilution of the FITC-conjugate concentrate that contains the binding protein conjugated to fluorescein, again washed twice in PBS, and incubated for 2 hr at 37°C at 1:500 dilution with a mitochondrial marker, MitoTracker red (Molecular Probes, Eugene, OR). After two washes in PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing the nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI), and observed under a fluorescent microscope using excitation and emission wavelengths of 365 and 490 nm, respectively (Olympus BH2, Melville, NY).

TUNEL assay. Frozen sections similar to those above were fixed and permeabilized as described previously (Henshall et al., 2000). Detection of DNA fragmentation was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique. In brief, tissue sections from control, Aβ(1–42)-treated and lithium/Aβ(1–42)-treated animals were dried for 15 min at room temperature and fixed in 10% formalin for 15 min, followed by a 10 min incubation in 1:2 vol/vol ethanol/acetic acid. Sections were washed three times in PBS for 5 min each, permeabilized with 3% Triton X-100 for 20 min and immersed in 3% hydrogen peroxide for 15 min. Sections were then washed three times in PBS buffer for 5 min each and processed for apoptosis detection using an Apoptosis Detection System, Fluorescein (Promega, Madison, WI). Five fields were captured from the CA1 region of the hippocampus from each animal, and results were compared between Aβ-treated and lithium/Aβ-treated animals. Results from the Aβ-treated rabbits were assigned a value of 100% and the lithium effect was expressed as the percent reduction in the number of positive neurons in the lithium/Aβ group when compared to the Aβ-treated rabbits.

Phosphorylated tau. Sections as above from control, Aβ(1–42)-treated and lithium/Aβ(1–42)-treated animals cut at the level of the hippocampus, were air-dried at room temperature, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of 1.5% normal serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse mAb against PHF tau (AT8) at 1:100 dilution. After washing with PBS and incubating with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin-biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining.

RESULTS

Lithium Treatment Prevents Aβ-Induced Endoplasmic Reticulum Stress

Procaspase-12 (~60 kDa) was detected primarily in the endoplasmic reticulum from all rabbits (controls, Aβ-treated and lithium/Aβ-treated), as shown in Figure 1A. In Aβ-treated animals, a cleavage product band (~40 kDa), corresponding to activated caspase-12, was released from the endoplasmic reticulum into the cytosol; treatment by lithium fully prevented this release. Calnexin mAb, a specific marker for endoplasmic reticulum, stained this subcellular fraction only (Fig. 1A). Illustrated in Figure 1B is the finding that procaspase-3/p32 is expressed mainly in the cytosolic fractions from control, Aβ-treated and lithium/Aβ-treated animals. Caspase-3 p17, an activated form of caspase-3, although not detectable in controls, was present as an intense band in the endoplasmic reticulum and to a lesser extent in the cytosol and nuclear fractions of Aβ-treated animals. Treatment with lithium prevented the formation of active caspase-3 p17.

Lithium Treatment Inhibits Aβ-Induced Mitochondrial Cytochrome C Release

Cytochrome c, localized chiefly in the mitochondrial-enriched fractions and to a lesser extent in the endoplasmic reticulum fractions in the controls, was released into the cytosol after Aβ administration; lithium treatment inhibited this Aβ-induced translocation of cytochrome c (Fig. 2A).

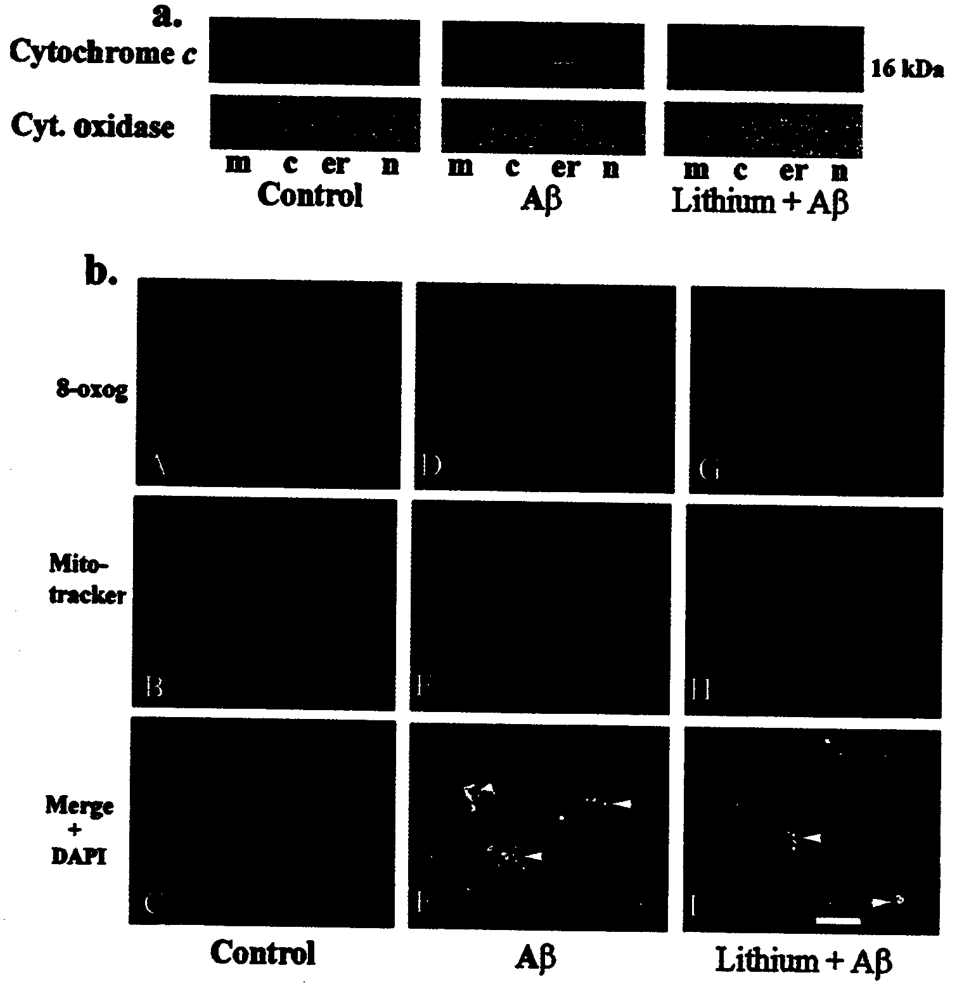

Fig. 2. Aβ induces mitochondrial stress. **A:** Cytochrome c, whereas detected in the mitochondrial (m) and to a lesser extent in the endoplasmic reticulum (er) fractions in controls, is present in the cytosolic (c) fraction after Aβ treatment; this translocation is largely prevented by lithium. Cytochrome c oxidase, used as marker for the mitochondria, stains only the mitochondrial fraction. **B:** Representative immunofluorescence photomicrograph at the mid-hippocampal level showing triple staining with an 8-oxoguanine mAb (green, A,D,G), which is a marker for oxidative DNA damage. MitoTracker (red, B,E,H) is used as a mitochondrial marker and DAPI (blue, C,F,I) for nuclei. Tissue from controls are depicted in the left panels (A–C), Aβ-treated in the center panels (D–F) and lithium/Aβ-treated in the right panels (G–I). In Aβ-treated animals, there is the emergence of positive staining for oxidative damage (D), an effect that is not prevented by lithium (G). Staining with 8-oxoguanine is primarily confined to mitochondria, represented as positive small round structures depicted by the arrows (F,I). Scale bar = 20 μm.

Cytochrome c oxidase subunit IV, used as a specific marker for mitochondria, confirmed the integrity of the mitochondrial fractions.

Lithium Treatment Does Not Inhibit Aβ Induced Oxidative Stress in Mitochondria

Whereas sections from controls did not exhibit 8-oxoguanine immunoreactivity (Fig. 2B, panel A), sections from Aβ-treated animals showed high immunoreactivity to this antibody (Fig. 2B, panel D) and lithium treatment did not prevent the Aβ-induced oxidative DNA damage (Fig. 2B, panel G). MitoTracker, applied as a mitochondrial marker (Fig. 2B, panels B, E, H), and DAPI as a nuclear marker (Fig. 2B, panels C, F, I) demonstrated that 8-oxoguanine immunoreactivity was localized predominantly in the mitochondria (small round foci) in Aβ-treated (Fig. 2B, panel F) and in lithium-treated (Fig. 2B, panel I) animals.

Lithium Treatment Decreases Aβ-Induced TUNEL Positivity

Sections from control animals treated with DNase I (positive control) showed widespread TUNEL positivity in the pyramidal layer (CA1) of the hippocampus (Fig. 3, panel A), whereas in a similar area in a representative section from the control animals there were no TUNEL-positive cells (Fig. 3, panel B). Aβ administration induced TUNEL positivity in this same region of the hippocampus (Fig. 3, panel C), which was reduced by lithium treatment (Fig. 3, panel D). Quantitative analysis of TUNEL positive cells at a magnification of 400× showed that the Aβ-induced TUNEL staining was reduced markedly by lithium treatment (Fig. 3, graph E).

Lithium Treatment Inhibits Aβ-Induced Translocation of NF-κB Into the Nucleus

The antibody to NF-κB p65 yielded two bands corresponding to p100 and p65, and the antibody to NF-κB p52 exhibited a single band; these were observed in the cytosolic fraction of controls, Aβ-treated and in lithium/Aβ-treated animals. Both gels exhibited all three bands, as seen in Figure 4. In Aβ-treated animals, the bands corresponding to p65 and p52 were also detected in the nuclear fractions, indicating NF-κB activation and translocation into the nucleus. In lithium-treated animals,

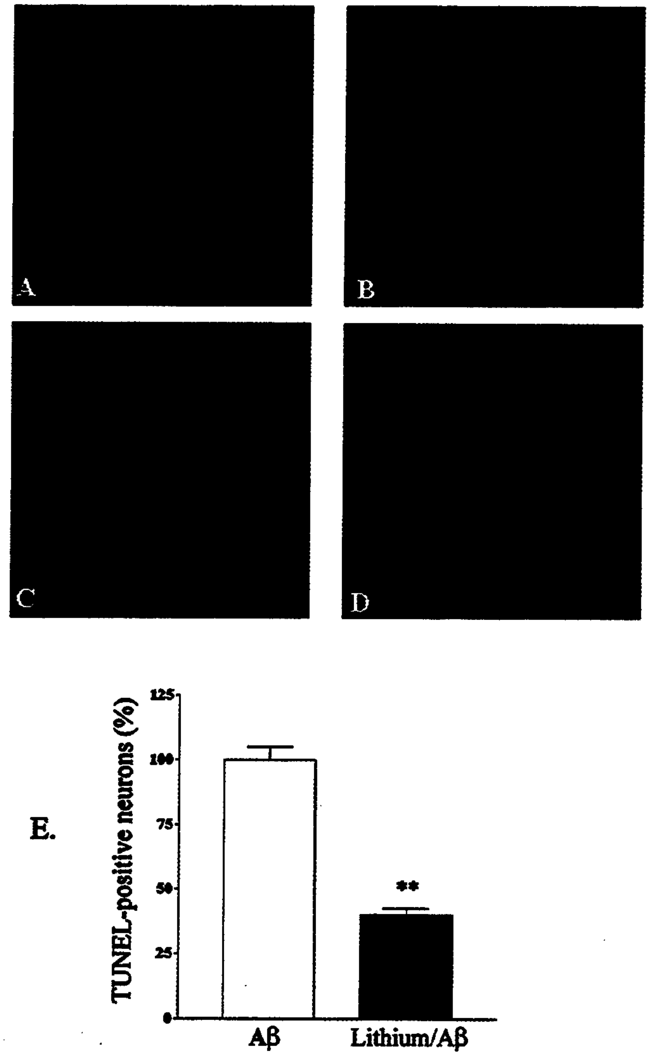

Fig. 3. Aβ-induced TUNEL positivity. A positive control section stained with DNase shows widespread TUNEL-positive staining in the pyramidal layer (CA1) of the hippocampus (**A**), whereas in a similar area in the control untreated group no TUNEL positive cells are seen (**B**). Aβ administration induces TUNEL-positive cells in the same region of the hippocampus (**C**) and lithium treatment reduces the emergence of this Aβ-induced TUNEL-positivity (**D**). Quantitative analysis (**E**), carried out on one section from each animal of the Aβ-treated and lithium/Aβ-treated animals, confirms the neuroprotective effect of lithium because there is a reduction in the number of TUNEL-positive neurons in comparison to Aβ-treated animals (panels A–D, 400×; $^{**}P < 0.01$, Student's *t*-test).

the p65 was barely detectable and the p52 band was reduced in comparison to Aβ-treated animals (Fig. 4).

Lithium Treatment Does Not Affect Aβ-Induced Phosphorylation of Tau

Immunohistochemical staining with the AT8 mAb demonstrated a lack of reactivity in control sections (Fig. 5A,D,G) but intense reactivity in the pyramidal cell layer of the hippocampus and the dentate gyrus after Aβ treatment (Fig. 5B,E,H), an effect that was not prevented by treatment with lithium (Fig. 5C,F,I).

Lithium Prevents Aβ-Induced Translocation of GSK-3 Into the Nucleus

Western blot analysis of GSK-3 in controls demonstrated two intense bands in the cytosolic fraction and two faint bands in the nuclear fraction corresponding to α and β isoforms (Fig. 6). After Aβ treatment, the nuclear bands increased markedly; lithium prevented the translocation of GSK-3β and fully abolished the presence of GSK-3α in the nucleus (Fig. 6). These treatments had no effect on GSK-3β phosphorylated at Ser^9 or Tyr^{216}.

DISCUSSION

Injection of aggregated Aβ into the brain of experimental animals may represent a valuable tool for studying the direct neurotoxic effect of this peptide. Intracerebral injection of Aβ has been demonstrated to produce dose-dependent cortical lesions in primates (McKee et al., 1998), and tau phosphorylation and neuronal loss in the common marmoset (Geula et al., 1998); however, these Aβ effects were not observed in younger animals or in rat brain. The use of rabbits may be relevant to investigations of AD pathogenesis to enhance information obtained from transgenic animals, aged primates and cell culture, because rabbits more closely resemble primates than they do rodents (Graur et al., 1996) and, like AD patients, develop eye-blink conditioning impairment (Woodruff-Pak and Trojanowski, 1996).

In the present study, Aβ(1–42) produces a wide variety of events, namely stress in the endoplasmic reticulum, oxidative damage at the mitochondrial level, and hyperphosphorylation of tau. Lithium treatment, although preventing the endoplasmic reticulum stress-evoked caspase-12 release and activation of caspase-3, has no preventative effect on oxidative mitochondrial DNA damage or on the hyperphosphorylation of tau.

Disturbances of endoplasmic reticulum function have been shown to trigger a distinct apoptotic pathway involving the activation of caspase-12 (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). Upon specific endoplasmic reticulum stress, procaspase-12, which resides in the endoplasmic reticulum, is released into the cytoplasm as active caspase-12. Once activated, caspase-12 may trigger the activation of effector caspases, such as caspase-3, and may initiate apoptosis. Studies by Nakagawa et al. (2000) and Nakagawa and Yuan (2000) with mouse cortical neurons have shown that Aβ(1–40) activates caspase-12, and that reduction in caspase-12 provides protection from apoptosis.

Our results demonstrate that the administration of Aβ(1–42) peptide also activates caspase-12, and cleaves the apoptosis initiator, caspase-3, within the endoplasmic reticulum. These results represent the first report describing the endoplasmic reticulum as the main site for active caspase-3 after Aβ administration in vivo, although we have recently reported that the endoplasmic reticulum is the major site of localization of active caspase-3 after the

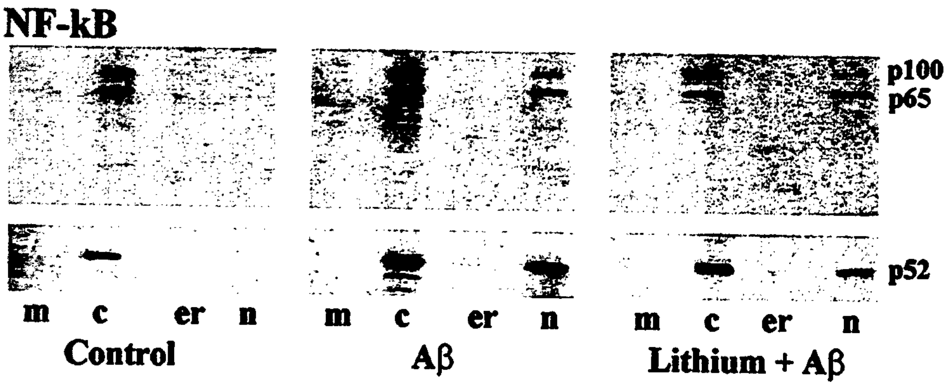

Fig. 4. Aβ-induced NF-κB activation. NF-κB is composed of p100, p65, and p52, and the corresponding bands are positive in the cytosolic fractions (c) from control, Aβ-treated, and lithium/Aβ-treated animals. Upon activation by Aβ, p65 and p52 partially translocate to the nucleus (n); lithium reduces translocation of p65 and, to a lesser extent, p52.

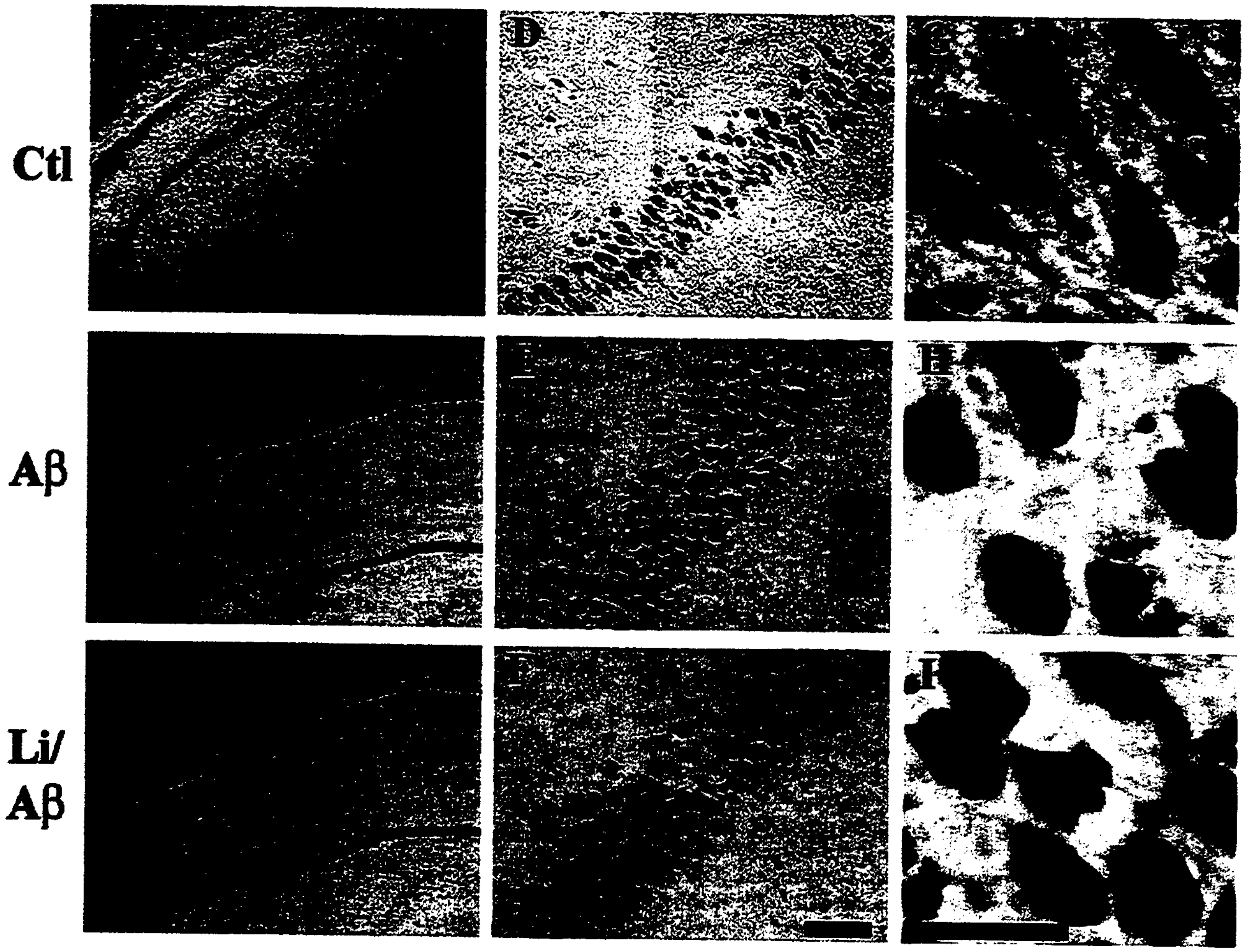

Fig. 5. Aβ-induces tau hyperphosphorylation. Photomicrographs of sections from the CA1 pyramidal cell layer of the hippocampus from control (**A,D,G**), Aβ-treated (**B,E,H**) and lithium/Aβ-treated (**C,F,I**) animals, stained with the AT8 mAb, which specifically detects hyperphosphorylated tau. No immunoreactivity is observed in the control; however, Aβ-treatment induces an intense immunoreactivity for hyperphosphorylated tau in the CA1 layer and dentate gyrus (B), and lithium treatment does not prevent this hyperphosphorylation from occurring (C). D–F: Photomicrographs of the CA1 pyramidal cell layer. G,H,I: Higher magnifications of a segment of this area. Scale bar = 40 μm (A–C); 50 μm (D–F); 10 μm (G–I).

intracisternal administration of the neurotoxin aluminum maltolate into rabbit brain (Ghribi et al., 2002a). In other studies, Aβ has been shown to induce caspase-3 activation and apoptosis in cerebral granule cells (Allen et al., 2001). Telencephalic neurons deficient in caspase-3 are not protected from Aβ-induced death, however, indicating that caspase-3 does not mediate Aβ neurotoxicity (Selznick et al., 2000). In another study, although Aβ robustly activates

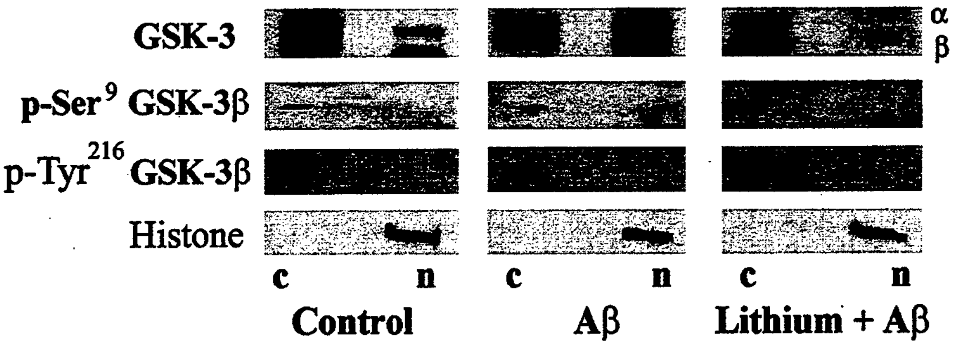

Fig. 6. Aβ induces translocation of GSK-3 into the nucleus. Cytosolic (c) and nuclear (n) fractions were separated from hippocampal tissue of control, Aβ-treated and lithium/Aβ-treated rabbits and Western blot analysis was carried out for GSK-3β (the mAb used also recognizes the α isoform), and for Tyr^{216} and p-Ser^9 GSK-3β. Aβ induces translocation of GSK-3 into the nucleus, which is fully prevented by lithium. In the control, p-Tyr^{216} GSK-3β and p-Ser^9 GSK-3β are confined to the cytosol and are not affected by Aβ or lithium treatment.

caspase-3 in rat cortical neurons, the Aβ neurotoxic effect is independent from this caspase activation, because caspase inhibitors fail to protect these neurons when exposed to Aβ (Saez-Valero et al., 2000).

The role of oxidative damage in the pathogenesis of AD has been examined in a number of studies (for review see Smith et al., 2000; Butterfield et al., 2001). Oxidative damage, as detected by an increase in the oxidized base 8-hydroxy-2′-deoxyguanosine (8-OHdG), has been found in postmortem AD brains (Gabbita et al., 1998; Lovell et al., 1999, 2000; Markesbery and Carney, 1999). Furthermore, mitochondrial DNA has been shown to be more sensitive to oxidative damage than nuclear DNA (Mecocci et al., 1994) and increased levels of 8-OHdG are accompanied by a decrease in mitochondrial 8-oxoguanine DNA glycosylase (hOGG1-2a), the enzyme that repairs 8-OHdG damage (Iida et al., 2002). Our results also demonstrate that damage to DNA occurs mainly in mitochondria.

The inducible transcription factor, NF-κB, is an important mediator of the human immune and inflammatory response, and increased levels of NF-κB activity have been observed in the brain of patients with various neurodegenerative disorders, including AD (Kaltschmidt et al., 1997). NF-κB has been demonstrated to promote neuronal survival in some cases, and in other cases to promote neuronal death. Inhibition of NF-κB may potentiate Aβ-mediated neuronal apoptosis (Kaltschmidt et al., 1999) whereas in other reports, NF-κB activation has been demonstrated to mediate Aβ-induced neurotoxicity (Bales et al., 1998). In global ischemia and traumatic spinal cord injury, NF-κB has been shown to promote neuronal death (for review see Mattson et al., 2000). Also, stress in the endoplasmic reticulum induced by a variety of stimuli may activate NF-κB (Guerrini et al., 1995; Pahl and Baeuerle, 1995; Pahl et al., 1996). The present study demonstrates that NF-κB is activated in response to the administration of Aβ(1–42). Whether translocation of NF-κB into the nucleus represents a cellular defensive or a deleterious mechanism after Aβ-induced ER stress and caspase-3 activation remains to be determined.

The controversy remains as to whether Aβ deposition or the accumulation of phosphorylated tau in NFTs is the primary cause of neurodegeneration in AD. The ability of Aβ to induce tau phosphorylation suggests that in AD, Aβ may lead to the generation of hyperphosphorylated tau and that tau accumulation may exacerbate the deleterious effect of Aβ; however, it has been demonstrated that tau phosphorylation is not associated with apoptosis in SH-SY-5Y human neuroblastoma cells treated with Aβ (Ekinci et al., 2000). The mechanism by which Aβ induces tau hyperphosphorylation remains unclear. GSK-3β is a physiological enzyme that has been considered as a candidate for tau phosphorylation and neurodegeneration in AD, and tau hyperphosphorylation in cultured cells induced by exposure to Aβ is attenuated by inhibition of GSK-3β activity (for review see Mudher and Lovestone, 2002). GSK-3β is subject to multiple regulatory mechanisms, among which phosphorylation has been the most widely studied. The activity of GSK-3β is increased by phosphorylation of Tyr^{216} (Wang et al., 1994; Hartigan and Johnson, 1999; Lesort et al., 1999); conversely, activity of GSK-3β is decreased by phosphorylation at Ser^9 (Plyte et al., 1992). In addition to regulation by phosphorylation, GSK-3β function can be regulated by the ability of GSK-3β to translocate from the cytoplasmic space into the nucleus. In the human neuroblastoma cell line, SH-SY-5Y, it has been demonstrated recently that nuclear levels of GSK-3β are increased rapidly after exposure of cells to different apoptotic stimuli, such as serum-free media, heat shock and staurosporine (Bijur and Jope, 2001). Interestingly, these treatments increase nuclear levels of GSK-3β, independent of phosphorylation at Ser^9 or Tyr^{216}.

We demonstrate that Aβ induces translocation of GSK-3β into the nucleus, and this translocation does not affect the p-Ser^9 GSK-3β or p-Tyr^{216} GSK-3β. Lithium treatment completely prevents the nuclear translocation of GSK-3β and has no effect on p-GSK-3β. Interestingly, inhibition of GSK-3β translocation by lithium is accompanied by inhibition of caspase-12 and caspase-3 activation at the endoplasmic reticulum level. These results are consistent with results obtained in the SH-SY5Y cell line showing that activation of caspases is a downstream event to the GSK-3β nuclear translocation, because caspase inhibitors do not prevent GSK-3β accumulation (Bijur and Jope, 2001). These results are relevant and may provide an explanation for the failure of caspase inhibitors to protect against the neurodegenerative effect induced by Aβ. Recently, a direct link between caspase-3 and GSK-3β has been demonstrated. Indeed, MPP^+ and rotenone, both inhibitors of mitochondrial complex I, induce caspase-3 activation; this effect was enhanced by increased GSK-3β activity and attenuated by inhibiting GSK-3β with lithium (King et al., 2001). In a second study in vascular smooth muscle cells and COS-7 cells, GSK-3 mediates hypoxia-induced apoptosis via the mitochondrial death pathway and inhibition of GSK-3 attenuated caspase-9 and caspase-3 activation (Loberg et al., 2002).

Inhibition of GSK-3β translocation by lithium fails to prevent the tau hyperphosphorylation evoked by Aβ. The failure of lithium treatment to reduce or prevent tau hyperphosphorylation after Aβ administration may be due to one or more of the following factors. First, lithium has been demonstrated to inhibit tau hyperphosphorylation (as recognized by AT8 reactivity) in a dose-dependent manner. Indeed, in non-neuronal COS cells transfected transiently with tau, lithium reduces the GSK-3β-induced tau phosphorylation only at a concentration of 1 mM or more (Sanchez et al., 2000). Therefore, the 7 mM lithium drinking water concentration used in the present study may yield a brain concentration less than the concentration needed to reduce tau phosphorylation; plasma concentrations of lithium with this dose are ~0.6 mM (Ghribi et al., 2002b). Because lithium has a narrow therapeutic index, higher concentrations of lithium may cause severe side effects, and administration of small molecules that directly inhibit GSK-3β may represent an alternative to the use of higher doses of lithium. In addition, other candidates for tau phosphorylation have been proposed. In cortical neurons and in SH-SY-5Y neuroblastoma cells, Aβ induced tau phosphorylation, an effect inhibited by a MAP kinase inhibitor (Ekinci et al., 1999). Furthermore, in the latter study by Ekinci et al. (1999), inhibition of MAP kinase activity also attenuated Aβ-induced reactive oxygen species and apoptosis. The MAP kinase family member, JNK, has been demonstrated to phosphorylate tau in vitro, and JNK has been shown recently to colocalize with tau deposits in AD (Atzori et al., 2001). There is also recent evidence that Aβ-induced neuronal death involves the activation of the JNK pathway (Troy et al., 2001) and similar to GSK-3β, JNK also phosphorylates tau (Reynolds et al., 1997, 2000). It is then possible that the JNK pathway activation plays a major role in tau phosphorylation. Further examination of the role of the JNK pathway in Aβ-induced mitochondrial oxidative stress and tau hyperphosphorylation is required.

In summary, the results reported here demonstrate that the direct administration of synthetic Aβ into rabbit brain induces stress in endoplasmic reticulum and mitochondria and promotes hyperphosphorylation of tau. Translocation of GSK-3β to the nucleus may represent an upstream event that leads to the activation of caspases, and may not be related to the Aβ-induced hyperphosphorylation of tau or to mitochondrial DNA damage; inhibition of GSK-3β redistribution by lithium prevents only the Aβ-induced caspase activation and does not reverse the Aβ-related mitochondrial DNA damage or the hyperphosphorylation of tau. Therefore, further studies are needed to determine the mechanisms that underlie Aβ damage to mitochondrial DNA and the hyperphosphorylation and accumulation of tau. Protection against the effects of Aβ may require, in addition to lithium, the use of agents that inhibit subsequent oxidative damage and tau hyperphosphorylation.

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APPENDIX II

Aβ(1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus.

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Abstract

We demonstrate that intracisternal injection of Aβ(1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These Aβ effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that Aβ-induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau.

Key words: Aβ, lithium, JNK, ERK, c-jun, tau.

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1. Introduction

The mechanisms by which neurons die in Alzheimer's disease (AD) are not fully understood. The observation that high levels of the soluble form of Aβ are found in the brain of AD patients suggests that the accumulation of this peptide plays a key role in the neuronal death that characterizes AD. However, the underlying mechanisms of this Aβ-induced neuronal death are still unclear. There have been a number of reports suggesting that activation of the MAP kinase signaling pathway plays an important role in mediating apoptotic neuronal death in AD (see for review [1]). In cultured rat microglia, treatment with 5 nM Aβ(25-35) is found to evoke the activation of the three MAP kinase subtypes, p38, ERK and JNK [2]. In mice transgenic for human amyloid precursor protein or containing a mutated presenilin-1 gene, both JNK and p38 are significantly activated in the cortex [3]. Mildly to severely affected AD patients also show activated JNK, ERK and p38 in the brain [4]. In addition to their putative role in regulating cell survival, there is mounting evidence that MAP kinases also phosphorylate tau protein (see for review [1]), the accumulation of which is a hallmark of AD. Taken together, these results indicate that the MAP kinase pathway is actively involved during the course of AD or experimentally *in vivo* or *in vitro* following the administration of synthetic Aβ.

We have recently shown that the intracisternal administration of Aβ(1-42) induces neuronal apoptosis and hyperphosphorylation of tau in the hippocampus of New Zealand white rabbits [5]. In this same study, we demonstrated that lithium inhibits apoptosis and fails to prevent the hyperphosphorylation of tau. Lithium is an inhibitor of GSK-3β, the activation of which is suggested to mediate Aβ-induced neurotoxicity. However, neither the mechanisms underlying the anti-apoptotic effect of lithium nor those triggering the phosphorylation of tau following Aβ treatment, are fully understood.

In the present study, we have tested the hypothesis that Aβ-induced apoptosis and phosphorylation of tau in rabbit hippocampus require activation of the MAP kinase signaling pathways. We have also examined the correlation of the anti-apoptotic effect of lithium, and its failure to prevent the phosphorylation of tau, to its effect on these signaling pathways.

2. Material and Methods:

Animals and treatment.

Adult (2-3 years and 4-5 kg) female New Zealand white rabbits received either intracisternal injections of 100 μL normal saline (n=6; controls), 100 μl of 2 mg/ml Aβ(1-42) (n=6; Aβ-treated group), 100 μL of 2 mg/ml Aβ(1-42) plus lithium (n=6; lithium/Aβ-treated group) or lithium alone (n=6; lithium-treated group). Aggregated Aβ(1-42) (American Peptide Company, Sunnyvale, CA) was prepared by incubating freshly solubilized Aβ(1-42) at a concentration of 2 mg/ml in saline at 37°C for 3 days. Lithium carbonate (Sigma Chemical Co, St. Louis, MO) was administered orally by adjusting the drinking water to a concentration of 7 mM as described previously [6].

Lithium was started 14 days prior to Aβ(1-42) administration and continued until animal sacrifice. Serum lithium concentrations were determined in blood specimens obtained from an ear vein at sacrifice, using an AVL model 9180 clinical analyzer (Roche Diagnostics, Roswell, GA). This oral lithium treatment resulted in serum lithium concentrations of 0.68 ± 0.32 mM/L (mean ±SEM). These levels are close to the range of 0.6-1.2 mM/L, concentrations that are achieved and maintained during lithium therapy in human subjects [7]. Rabbits were sacrificed 7 days after the intracisternal administration of Aβ(1-42) or saline, and at necropsy were perfused with Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY) at room temperature. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Western blot analysis.

Tissue from the entire hippocampus was gently homogenized using a teflon homogenizer (Thomas, Philadelphia PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/mL pepstatin and 12.5 mg/mL of N-acetyl-Leu-Leu-Norleu-Al). Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Illinois). Proteins (10 μg) were separated by SDS-PAGE (10 % gel), followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and were incubated overnight at 4° C with mouse monoclonal antibody (mAb) to JNK1/2, p-JNK1/2, ERK1/2, p-ERK1/2, c-fos, p-c-jun (Santa Cruz Laboratories, Santa Cruz, CA), p38 and p-p38 (Cell Signaling, Beverly, MA), and to caspase-3 (Active Motif, Carlsbad, CA). Phosphorylation of the microtubule-associated protein tau (tau) was assessed using either a 1:1000 dilution of the AT8 mAb (Innuogenetics, Ghent, Belgium) which recognizes a doubly phosphorylated epitope including S^{199}, S^{202}, and T^{205}. We also used mAb PHF-1 at a 1: 2500 dilution which also reacts with tau (gift from Dr. Peter Davis, Albert Einstein College of Medicine). β-actin (Sigma, Saint Louis, MI) was applied as a gel loading control at a 1:500 dilution. The blots were developed with enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA) and bands were analyzed by densitometry with Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

TUNEL assay.

Apoptosis detection was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique on frozen coronal brain sections (14 μm thick) from the mid-hippocampal level of control, Aβ-treated, lithium/Aβ-treated, and lithium-treated animals. Detection of DNA fragmentation was performed using the Apoptosis Detection System (Fluorescein, Promega, Madison, WI) as we previously have reported [5;6]. Sections were counterstained with propidium iodide, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a fluorescent microscope using excitation/emission wavelengths of 365/490 nm (Olympus BH2, Melville, NY).

3. Results:

Lithium inhibits Aβ-induced apoptosis

Western blot analysis with caspase-3 mAb (Figure 1a) shows a 32 kDa band that corresponds to pro-caspase-3 in control, Aβ-treated, lithium/Aβ-treated, and lithium-treated animals. Caspase-3 p17 and p12, two activated forms of caspase-3, are present in the Aβ-treated animals only. Treatment with lithium completely inhibits cleavage of pro-caspase-3 to the active caspase-3 p 17 and p12.

Application of the TUNEL assay (Figure 1b), examined in the pyramidal layer (CA1) of the hippocampus, shows that while sections from control animals (panel A) exhibit no TUNEL-positive neurons, the Aβ-treated animals have numerous TUNEL-positive neurons (panel B); administration of lithium greatly reduces the TUNEL positivity (panel C). Treatment by lithium alone does not induce TUNEL positivity (panel D).

Aβ(1-42) induces phosphorylation of JNK and ERK, but not of p38, effects that are differentially regulated by lithium

The JNK mAb used in these studies reacts with both JNK1 and 2 and yields 54 and 46 kDa bands; the mAb recognizing phosphorylated JNK (p-JNK) also is immunoreactive with both p-JNK1 and p-JNK2 (Figure 2a). In all animal groups, levels of the 46 kDa JNK are higher than those of 54 kDa JNK. Administration of Aβ peptide significantly raises the levels of p-JNK; lithium inhibits the Aβ-induced increase in p-JNK and has no significant effect when administered alone (Figures 2a and b). While p-c-jun is not detected in controls, it is expressed following Aβ(1-42) administration; lithium treatment prevents p-c-jun expression (Figure 2a).

Both 42 and 44 kDa ERK (Figure 3a) are detected in hippocampal tissue from control, Aβ-treated, lithium/Aβ-treated, and lithium-treated animals. Administration of Aβ(1-42) induces activation of p-ERK and this activation increases following treatment by lithium. Treatment with lithium alone significantly increases the levels of p-ERK 42 kDa compared to untreated controls; this treatment induces activation of lower levels of the 44 kDa band (Figure 3a and b). The transcription factor c-fos is not detected in controls but is expressed following Aβ; lithium increases the levels of c-fos either when combined with Aβ or when administered alone (Figure 3 a).

p38 protein is present at approximately the same level in the hippocampus of control, Aβ-treated, lithium/Aβ-treated, and lithium-treated animals (Figure 3c). Phosphorylated p38 (p-p38) is barely detectable in these various treatment groups and does not appear to be affected appreciably by any of these treatment regimes, as depicted in Figure 3c.

Lithium doesn't prevent Aβ-induced p-tau

Aβ treatment results in the phosphorylation of the microtubule-associated protein tau as demonstrated by mAb AT8 or PHF-1 immunostaining (Figure 4). This Aβ-induced phosphorylation is not affected by lithium treatment, and lithium alone does not induce phosphorylation of tau.

4. Discussion:

The present study demonstrates that the intracisternal administration of synthetic Aβ(1-42) peptide into rabbit brain induces apoptosis, as indicated by the activation of caspase-3 and TUNEL, and by the phosphorylation of tau demonstrated by AT8 and PHF-1 immunoreactivity. These effects correlate with the activation of the MAP kinase members, JNK and ERK, and their respective substrates, c-jun and c-fos. Aβ does not activate the third MAP kinase family member, p38. Lithium treatment prevents the activation of apoptosis but not the hyperphosphorylation of tau. The ability of lithium to prevent apoptosis and its failure to prevent the phosphorylation of tau following Aβ administration correlates with the inhibition of JNK and its substrate c-jun, and with the increase in ERK phosphorylation and expression of its substrate c-fos. Interestingly, lithium administered alone activates ERK and c-fos, however, this activation is not associated with the induction of apoptosis or the phosphorylation of tau.

Cultured cells exposed to synthetic Aβ have demonstrated neurodegeneration and hyperphosphorylation of tau [8-10]. The mechanism by which Aβ induces tau hyperphosphorylation has been suggested to involve the activation of GSK-3β [11];[9]. Lithium has been shown to be neuroprotective against a variety of experimental neurotoxic stimuli [12] and to prevent tau hyperphosphorylation [13] by mechanisms that are suggested to be mediated via the GSK-3β signaling pathway. However, as we have shown recently [5] and in the present study, the failure of lithium to prevent hyperphosphorylation of tau suggests that GSK-3 β phosphorylation is not involved in the Aβ-induced accumulation of tau in our model system. Many other enzymes have been suggested to phosphorylate tau. Apart from regulating apoptosis, MAP kinases also can phosphorylate neurofilaments and proteins including tau protein [14-16].

Our results, showing that Aβ activates JNK and ERK, raise the possibility that JNK or ERK may be responsible for the Aβ-evoked phosphorylation of tau. However, inhibition of JNK by lithium is not sufficient to prevent the phosphorylation of tau. Furthermore, activation of ERK by lithium alone does not induce the phosphorylation of tau. These results strongly suggest that phosphorylation of tau by Aβ in our model system is independent of the activation of the MAP kinase members.

The anti-apoptotic effect we report in the present study with lithium is in accordance with recent data showing that pretreatment for 7 days with 0.5-2 mM of lithium has prevented the glutamate-induced activation of JNK and c-jun, and the induction of apoptosis in granule cells [17]. However, in another study, lithium has been demonstrated to increase p-c jun and p-JNK in SH-SY5Y cells and in rat brain [18]. Valproic acid, which like lithium is a mood stabilizer, promotes neurite growth and cell survival by mechanisms that correlate with the activation of ERK kinase [19]. Activation of JNK and its substrate c-jun following Aβ treatment, and their inhibition by lithium, suggest that JNK and c-jun may be involved in regulating apoptosis in our animal system. In contrast, the observation that lithium increases ERK activation and c-fos levels without the induction of caspase-3 cleavage may suggest that ERK activation is not involved in regulating apoptosis. The mechanism that underlies c-fos activation and the significance of this activation remain to be determined. The role of ERK in Aβ toxicity and phosphorylation of tau has been controversial. Activated ERK has been shown to be associated with early tau deposition in neurodegenerative diseases [20], and the downregulation of ERK has been associated with elevated Aβ [21]. In another study, however, it was demonstrated that ERK activation did not mediate Aβ neurotoxicity in rat cortical neurons [22]. Our results demonstrate that an increase in ERK activity by lithium alone does not correlate with the induction of apoptosis or the phosphorylation of tau, suggesting that ERK does not mediate the process of Aβ toxicity in our animal model. Further studies using specific inhibitors of JNK and ERK will better define the role of these MAP kinase family members in the phosphorylation of tau and the regulation of apoptosis following Aβ administration.

Acknowledgment: Supported by the United States Department of Defense and the Virginia Center on Aging. We thank Dr. Peter Davis for his gift of the PHF antibody.

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Legends for Figures:

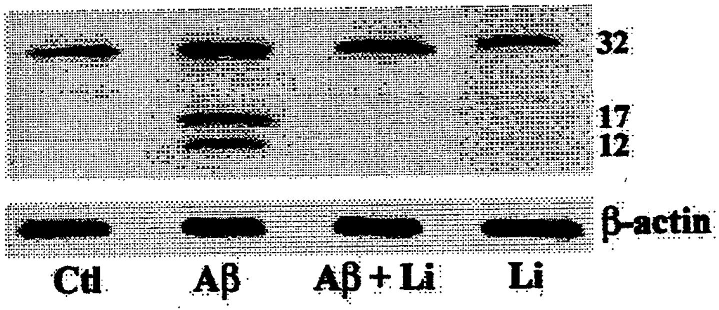
Figure 1. a. Aβ induces cleavage of the pro-caspase-3 (32 KDa) to p17 and p12. This cleavage is prevented by lithium. **b.** Immunofluorescence images of TUNEL labeling in the hippocampal CA1 area. Control section shows no TUNEL-positive neurons (panel A) while a section from an Aβ-treated animal shows widespread DNA fragmentation (arrows, panel B). Treatment with lithium reduces the number of neurons exhibiting DNA fragmentation (panel C) and no TUNEL labeling is seen with lithium alone (panel D). Sections were counterstained with propidium iodide (red).

Figure 2. a. Representative Western blots of JNK, p-JNK and p-c-jun from hippocampal tissue. JNK is detected as a 54 kDa band and a more intense band at 46 kDa in the various groups. p-JNK is also detected as 54 and 46 bands and is increased by Aβ and reduced by lithium. Lithium alone does not increase p-JNK. p- c-jun is not detected in the control but is expressed following Aβ(1-42) administration and is markedly reduced by lithium. **b.** Densitometric scanning analysis showing that lithium markedly reduces the Aβ-induced increase in p-JNK **$p<0.01$ vs control, ++$p<0.01$ vs Aβ (ANOVA followed by Student *t* test).

Figure 3. a. Representative Western blots of ERK, p-ERK and c-fos. ERK is detected as a double band at 44 and 42kDa. The active p-ERK is also detected as a double band at 44 and 42 kDa, which is barely seen in controls, but is significantly elevated following Aβ)1-42). Lithium increases the intensity of p-ERK when administered with Aβ(1-42) or alone. No c-fos is detected in the control but is induced by Aβ(1-42) treatment. Lithium slightly increases the level of c-fos in comparison to the Aβ(1-42)-treated animal. **b.** Densitometric analysis of p-ERK from hippocampal tissue shows that lithium with Aβ(1-42) increases the levels of p-ERK in comparison to the animal treated with Aβ(1-42) alone, or to the control. $^*p<0.05$ vs control, $+p<0.05$ vs Aβ. **c.** p38 is distributed at similar levels in all of the groups, while the phosphorylated form (p-p38) is barely detectable.

Figure 4. AT8 and PHF-1 mAbs only faintly stain hippocampal tissue in a control but in the Aβ-treated animal there is intense immunoreactivity which is not affected by lithium treatment. No AT8 or PHF-1 positive bands are seen following treatment with lithium alone.

a. Caspase-3


32
17
12
β-actin
Ctl
Aβ
Aβ + Li
Li

b. TUNEL

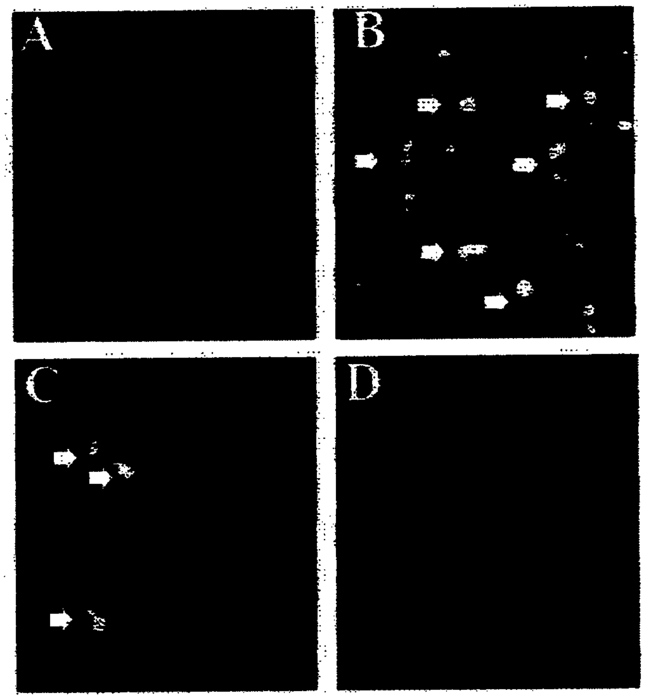


Fig 1.

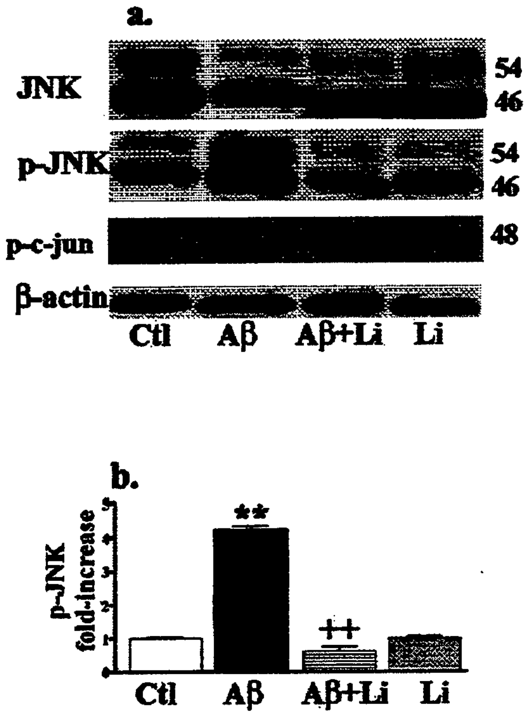

a.
JNK
p-JNK
p-c-jun
β-actin
54
46
54
46
48
Ctl
Aβ
Aβ+Li
Li
b.
p-JNK fold-increase
0
1
2
3
4
5
**
++
Ctl
Aβ
Aβ+Li
Li

Fig.2

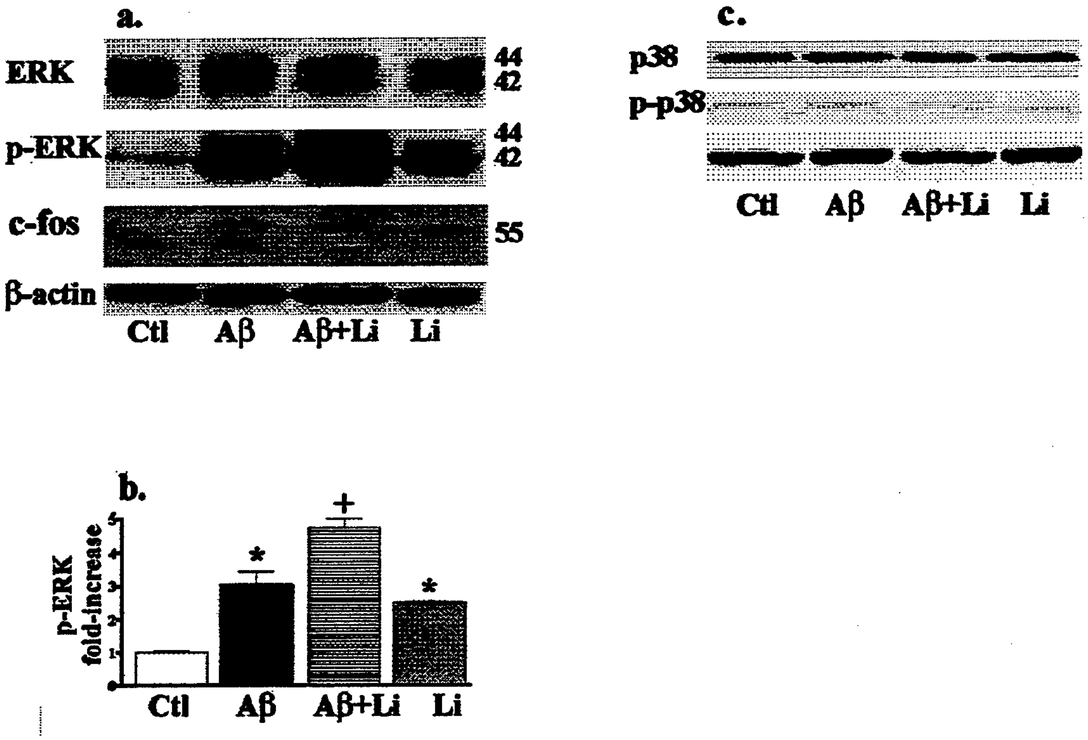

a.
ERK
44
42
p-ERK
44
42
c-fos
55
β-actin
Ctl
Aβ
Aβ+Li
Li
c.
p38
p-p38
Ctl
Aβ
Aβ+Li
Li
b.
p-ERK fold-increase
0
1
2
3
4
5
*
+
*
Ctl
Aβ
Aβ+Li
Li

Fig.3

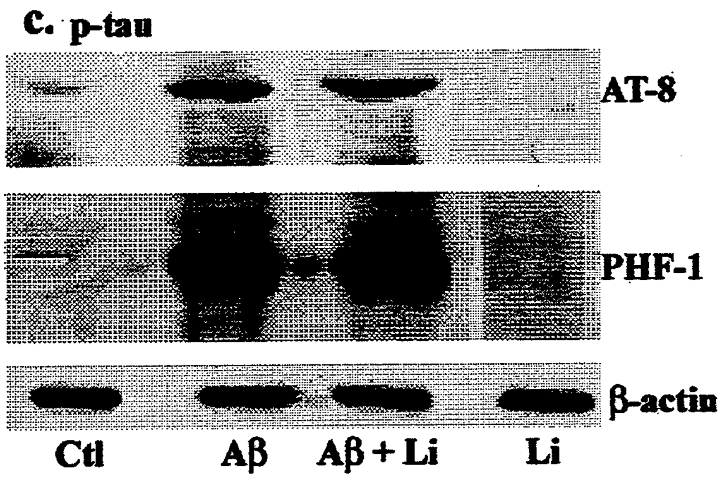

c. p-tau
AT-8
PHF-1
β-actin
Ctl
Aβ
Aβ + Li
Li

Fig.4

APPENDIX III

Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain.

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Key Words: aluminum, apoptosis, caspase-3, caspase-12, gadd 153, endoplasmic reticulum, lithium.

Abstract

Loss of neurons is a hallmark of neurodegenerative disorders and there is increasing evidence suggesting that apoptosis is a key mechanism by which neurons die in these diseases. Mitochondrial dysfunction has been implicated in this process of neuronal cell death but there is a growing body of evidence suggesting also an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondria, or in concert with mitochondrial-initiated pathways. Investigations in our laboratory have focused on neuronal injury resulting from the administration of aluminum maltolate, via the intracisternal route, to New Zealand white rabbits. Treatment of rabbits with aluminum maltolate induces both mitochondrial and endoplasmic reticulum stress. Agents such as lithium or GDNF, have the ability to prevent aluminum-induced neuronal death by interfering with the mitochondrial and/or the endoplasmic reticulum-mediated apoptosis cascade. This animal model system involving neurotoxicity induced by an aluminum compound, provides new information on mechanisms of neurodegeneration and neuroprotection.

1. Introduction

Aluminum (Al) is ubiquitous in the environment and it has been speculated that its accumulation in human brain might be linked to neurodegenerative disorders including Alzheimer's disease, the parkinsonism-dementia complex of Guam and amyotrophic lateral sclerosis (see for review [1]). There are numerous reports both supporting and denouncing this link (see for review [2]) and further work is needed to clarify the possible role, if any, of Al in these devastating human disorders. Aluminum is unquestionably neurotoxic as was demonstrated by Alfrey et al., who discovered that Al accumulation was the cause of a fatal encephalopathy in many patients on longterm hemodialysis treatment for chronic renal failure [3]. There is also strong evidence implicating Al as a potent neurotoxin when it accumulates at a certain level in animal brain or in cultured cells and neurons. However, the molecular mechanisms by which Al interacts with cells and neurons and induces its neurotoxicity are poorly understood.

Some of the cellular process that are involved in the neurodegeneration induced by Al have been clarified in our laboratory by studies on New Zealand white rabbits treated with Al maltolate via the intracisternal route of administration. A review of our findings forms the basis of this present report.

2. Effect of Al on the mitochondrial-mediated apoptosis pathway

Apoptosis, or programmed cell death, plays a critical role in the normal development and maintenance of tissue homeostasis, and is also a process by which brain cells die in neurotoxic situations. Mitochondrial changes following cytotoxic stimuli represent a primary event in apoptotic cell death. The apoptogenic factor, cytochrome *c*, is released from mitochondria into the cytoplasm where it binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspase, caspase-3. Release of cytochrome *c* from the mitochondria has been shown to involve three distinct pathways. One implicates the opening of the mitochondrial permeability transition pore (MTP), the second is triggered by the translocation to mitochondria of the pro-apoptogenic Bax which can form a channel by itself, and the third may result from the interaction of Bax with the voltage-dependent anion channel (VDAC) to form a larger channel which is permeable to cytochrome *c*. In contrast to Bax, the anti-apoptotic Bcl-2 has the ability to block the release of cytochrome *c* from mitochondria by mechanisms such as a direct blockade of the MTP opening, or by functioning as a docking protein [4,5].

Al has been demonstrated to accumulate in neurons following cell depolarization, where it inhibits Na^+/Ca^{+2} exchange and thereby induces an excessive accumulation of mitochondrial Ca^{+2} [6]. Increases in intramitochondrial Ca^{+2} levels lead to an opening of the MTP, with cytochrome *c* release and subsequent apoptosis resulting from activation of the caspase family of proteases.

We have shown that the intracisternal administration of Al maltolate results in cytoplasmic cytochrome *c* translocation, Bcl-2 down-regulation and Bax up-regulation. as well as caspase-3 activation [7]. These results indicate that Al targets the mitochondria. Furthermore, the fact that we have demonstrated that the release of cytochrome *c* is inhibited by cyclosporin A, a specific inhibitor of the MTP opening, implicates opening of the MTP as the process by which cytochrome *c* translocates to the cytoplasmic space from mitochondria [8]. The mitochondria-regulated death pathway has also been shown to mediate asbestos-induced apoptosis in alveolar epithelial T cells [9], and iron has been demonstrated to alter Bcl-2 levels in mitochondria and to affect the MTP [10].

The use of pharmacological agents that prevent or reverse the apoptotic effects of Al can provide valuable mechanistic information on the effects of Al on cellular protein targets. We have demonstrated that the glial cell-line derived neurotrophic factor (GDNF) protects rabbit hippocampus from the neurotoxic effect of Al, but does not prevent the release of cytochrome *c* [11]. These results do not support the release of cytochrome *c* as the sole trigger of aluminum-induced apoptosis, at least in this animal model system. However, GDNF treatment increases the level of the anti-apoptotic protein, Bcl-XL, which when over-expressed, has the ability to sequester Apaf-1, and thereby to inhibit Apaf-1-dependent caspase-9 activation [12,13]. Formation of the cytochrome *c*-Apaf-1 complex, rather than the presence *per se* of cytochrome *c* in thecytoplasm, thus appears to be the determining factor in the activation of caspases and triggering of apoptosis.

The neuroprotective effect of GDNF treatment resulted from a single intracisternal injection of GDNF. We have shown that chronic treatment of rabbits with lithium in the drinking water results in inhibition of the Al-induced cytochrome *c* release, enhances levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL, prevents the redistribution of the pro-apoptotic protein Bax levels, and inhibits caspase-3 activation and DNA fragmentation [14]. The ability of lithium to block the release of cytochrome *c* might be attributed to either a direct effect on mitochondria by inhibiting the opening of the MTP, Bax translocation, or to an increase in the level of the anti-apoptotic Bcl-2. However, it is unlikely that lithium possesses the ability to directly block the opening of the MTP, as is the case for cyclosporin A which blocks this channel by specifically binding its matrix protein, cyclophilin D [15,16]. Rather, lithium may inhibit cytochrome *c* translocation by preventing (i) an Al-induced redistribution of Bax and (ii) a decrease in Bcl-2. Lithium possesses neuroprotective properties since it has been demonstrated to exert robust protective effects against diverse apoptotic insults induced by potassium deprivation [17], glutamate [18], β-amyloid [19,20], ischemia and anticonvulsant-induced apoptosis [21,22], and staurosporine and heat-shock [23]. One of the molecular mechanisms by which lithium protects against neuronal death has been attributed to the inhibition of glycogen synthase kinase-3 (GSK-3) [23], an enzyme which when activated promotes pro-apoptotic signaling [24]. Lithium has been shown to inhibit caspase-3 activation and to protect cerebellar granule cells from apoptosis induced by potassium deprivation [25] and to robustly increase levels of the anti-apoptotic Bcl-2 in various brain regions in rats and mice [18,26].

It appears from our studies of neurotoxicity in rabbits that Al induces perturbation of mitochondrial function, affecting the distribution and levels of the apoptosis regulatory proteins, and triggering signaling that leads to the initiation and expression of apoptosis. However, it remains unclear whether Al directly gains access to mitochondria or triggers mitochondrial stress through other signaling pathways.

Effect of aluminum on apoptosis-regulatory proteins that mediate endoplasmic reticulum stress

Although mitochondrial alterations may represent an important step in the mechanisms underlying neuronal cell death induced by Al, studies in our laboratory have provided evidence suggesting that the endoplasmic reticulum (ER) also plays an important role in regulating this cell death. The ER is an important subcellular site, since it is the major storage location for calcium and contains members of the *Bcl-2* family of proteins, Bcl-2 and Bcl-XL.

Endoplasmic reticulum stress-inducing agents have been shown to activate the expression of various genes, such as those coding for the gene *gadd 153*, important in growth arrest and DNA damage-induction, and the inducible transcription factor, NF-κB, which plays an important role in the survival of neurons. Stress induced in the ER has also been shown to result in a specific type of apoptosis mediated by caspase-12 and is independent of mitochondrial-targeted apoptotic signals. Also, the ER can work in concert with mitochondria to regulate cellular apoptosis. Indeed, it has been reported that the drug brefeldin induces ER dilatation and leads to cytochrome *c* release and caspase-3 activation [27]. This effect can be blocked by wild type Bcl-2 and, surprisingly, a Bcl-2 variant that is exclusively targeted to the ER, is also able to accomplish the same task. Bcl-2 may exert its protective effect by controlling calcium homeostasis in the ER as well as the mitochondria.

Studies performed in our laboratory have shown that Al-maltolate induces a redistribution of the apoptosis-regulatory proteins, with Bax being present at higher levels in the ER than in thecytosol and with decreased amounts of Bcl -2 in the ER [7]. We also have shown that Al induces stress in the ER, as demonstrated by the activation of *gadd 153* and its translocation into the nucleus. The *gadd 153* gene is specifically activated by agents that disturb ER function. We have also provided the first report of the *in vivo* activation of caspase-12 following Al treatment [28].

Although we have demonstrated an effect of Al on ER function, it remains unclear which signaling mechanisms lead to perturbation of ER homeostatsis by Al. Aluminum may disturb Ca^{2+} homeostasis or protein processing in the ER. Stress signaling from the ER is suggested to be triggered by two distinct pathways, the unfolded protein response (UPR) and the overload response (EOR), for review see [29]. Severe insult that results in sustained depletion of Ca^{2+} stores or to prolonged ER stress leads to apoptotic cell death. In accordance with our hypothesis are data from cultured rat hippocampal cells treated with Al chloride showing the induction of cell death, a pathologic process diminished by treatment with dantrolene, a known inhibitor of Ca^{2+} release from intracellular stores [30].

Conclusions

Speculation that apoptosis underlies Al-induced neuronal cell loss is gaining increased attention in the recent literature. Our earlier research primarily involved immunohistochemistry for demonstrating the toxic effects of the intracisternal administration of Al maltolate. We have extended this work by using subcellular fractionation of brain tissue and Western blot analysis to study markers of apoptosis. We have shown that the ER is a key organelle in regulating apoptosis and we have provided the first evidence for the *in vivo* activation of caspase-12. We have also demonstrated for the first time that activated caspase-3 is localized in the ER as well as in the cytoplasm and nucleus.

Our overall hypothesis (see Figure) is that Al induces ER stress with a response that leads to cross talk between the ER and mitochondria, eventually culminating in the activation of caspases and apoptosis. Stress in the ER results in perturbation of Ca^{2+} homeostatsis, leading to down-regulation of the ER chaperones and anti-apoptotic proteins, up-regulation of the pro-apoptotic proteins, and activation of caspase-12. An increase in extracellular Ca^{2+} subsequent to ER stress leads to an increase in mitochondrial Ca2+. The increase in mitochondrial Ca^{2+} results in depolarization of mitochondria, release of cytochrome *c* and activation of caspase-9. Activation of caspase-12 by ER stress and of caspase-9 by mitochondrial stress in turn activates the effector caspase-3, with the eventual induction of apoptosis. We suggest that maintaining proper functioning of the ER, using pharmacological agents which prevent the perturbation of ER-Ca^{2+} mechanisms, represents an important novel approach for the prevention of Al-induced neuronal injury.

Acknowledgment.

Supported by Grant # DAMD 17-99-1-9552 from the US Department of the Army and the Virginia Center on Aging.

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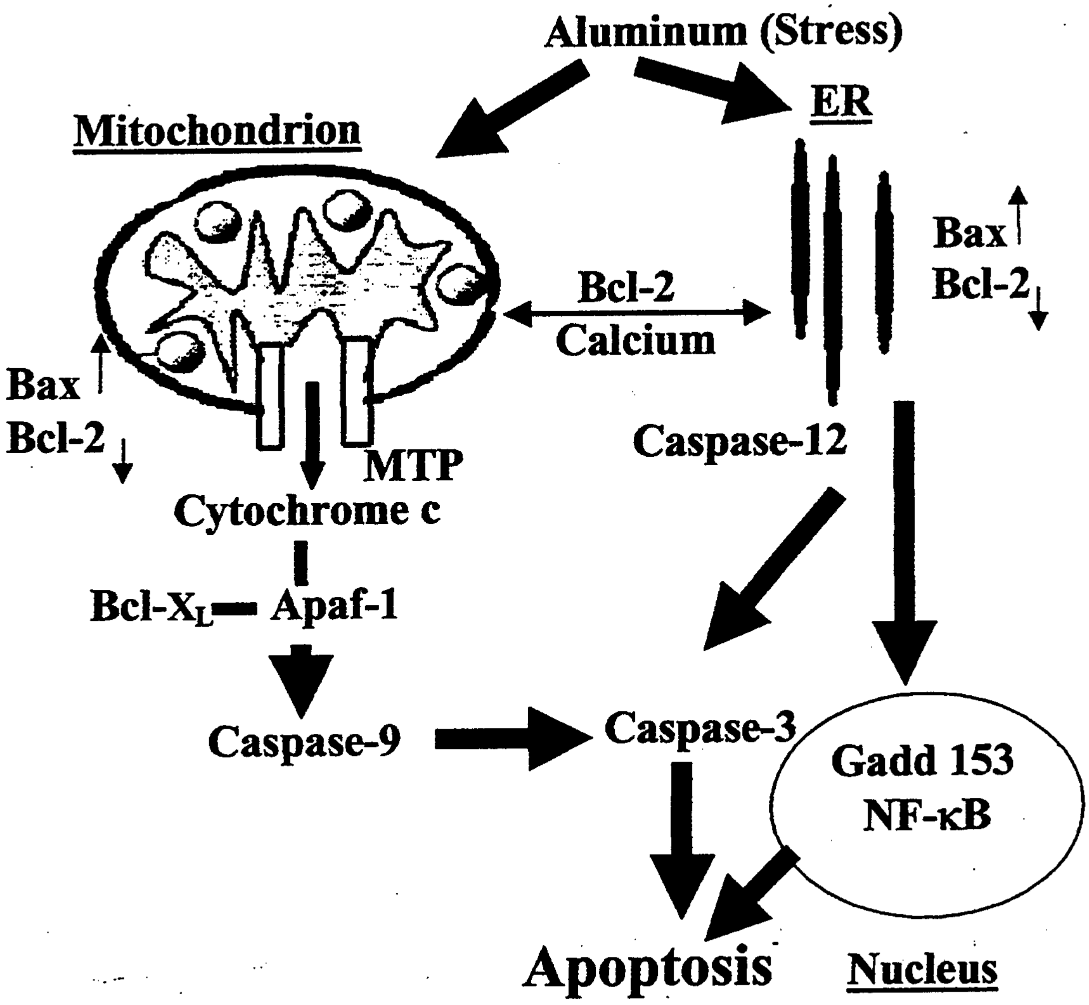
Legends for Figures

Figure 1:

Aluminum induces neuronal apoptosis by its effects on the functioning of both the endoplasmic reticulum and mitochondria. At the endoplasmic reticulum level, Al leads to activation of caspase-12, which in turn can activate the effector caspase-3. Al-induced endoplasmic reticulum stress can also activate the transcription factors *gadd 153* and NF-κB, leading to their translocation into the nucleus where they initiate apoptosis. Stress in mitochondria leads to opening of the mitochondria permeability transition pore , release of cytochrome *c* activation of caspase-9 and of caspase-3. Both stress in mitochondria and in the endoplasmic reticulum leads to down-regulation of the anti apoptotic proteins Bcl-2 and increase in the levels of the pro-apoptotic Bax and activation of the effector of apoptosis, caspase-3. Bcl-X_L, another anti-apoptotic protein, has the ability when overexpressed in the cytosol to sequester Apaf-1, and thereby to inhibit cytochrome c-Apaf-1 dependent caspase-9 activation. Stress in the endoplasmic reticulum may also lead to perturbation of the Ca^{2+} stores and of the correct folding of proteins resulting in an increase in cytosolic Ca^{2+} concentrations. Subsequent to this increase in cytosolic Ca^{2+} levels, mitochondrial Ca^{2+} also rises. As mitochondria cannot retain more Ca^{2+} than what is required, the excess Ca^{2+} is released back into the cytosol, a consequence of which is the opening of the MTP and release of cytochrome *c*. This cross-talk between mitochondria and endoplasmic reticulum involving Ca^{2+} is suggested to be regulated by Bcl-2.


Aluminum (Stress)
Mitochondrion
ER
Bax
Bcl-2
Bcl-2
Calcium
Bax
Bcl-2
MTP
Cytochrome c
Caspase-12
Bcl-X$_L$
Apaf-1
Caspase-9
Caspase-3
Gadd 153
NF-κB
Apoptosis
Nucleus

APPENDIX IV

Abstract

Aluminum-induction of endoplasmic reticulum-specific apoptosis in experimental neurodegeneration and its reversal by lithium. John Savory[1,3] , Othman Ghribi[1], Mary M. Herman[2] . *Depts. of Pathology[1] and Biochemistry and Molecular Genetics[3], University of Virginia and IRP[2], NIMH, NIH.*

Although apoptosis under mitochondrial control has received considerable attention, mechanisms utilized within the endoplasmic reticulum (ER) and the nucleus in mediating apoptotic signals are not well understood. However, there is evidence in the literature suggesting that the ER plays an active role in regulating apoptosis. We have injected aluminum (Al) maltolate intracisternally into aged rabbits and have observed the induction of nuclear translocation of *gadd 153* and of the inducible transcription factor, NF-κB. Translocation of these two proteins is accompanied by decreased levels of Bcl-2 in both the ER and nucleus. This treatment also induces caspase-12 activation which is a mediator of ER-specific apoptosis. These studies have been extended to assess neuroprotective strategies. Treatment with 7 mM of lithium carbonate in drinking water prevents the Al-induced translocation of cytochrome *c*, up-regulates Bcl-2 and Bcl-X_L, down-regulates Bax, abolishes caspase-3 activity and dramatically reduces DNA damage. The regulatory effect of lithium on the apoptosis-controlling proteins occurs in both the mitochondria and the ER. Thus, lithium offers promise for the treatment of neurodegenerative disorders since it is a relatively safe agent, can be administered orally, and has been used therapeutically for several decades.

APPENDIX V

MPP$^+$ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF-κB signaling pathways.

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ABSTRACT.

Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie the MPTP/MPP$^+$-induced neurotoxicity, which has been used extensively as a model for Parkinson's disease. In the present study we have examined the hypothesis that MPP$^+$ targets the endoplasmic reticulum. Because rabbits possess more genetic similarities to primates than to rodents we have selected this animal model system for our MPP$^+$ neurotoxicity studies. MPP$^+$ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. Here we demonstrate that MPP$^+$ in a dose-dependent manner induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by activation of ATF-6 and NF-κB, leads to activation of gadd 153. These effects correlate with the activation of caspase-3 and of JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

KEY WORDS. MPP$^+$, endoplasmic reticulum, ATF-6, NF-κB, gadd 153, caspase-3, JNK.

INTRODUCTION.

MPTP is a pro-toxin converted by monoamine oxidase B to the active toxin, MPP$^+$. MPTP/MPP$^+$ is widely used to model Parkinson's disease in non-human primates and rodents, in which it induces a selective loss of dopaminergic neurons in the substantia nigra. MPP$^+$ is selectively accumulated by dopaminergic neurons and is concentrated within mitochondria where it acts to inhibit complex I of the electron transport chain. This effect results in a decline in the production of ATP and an increase in the formation of reactive oxygen species. These events are suggested, ultimately, to lead to cell death (for review see (1, 2). Although mitochondrial involvement in Parkinson's disease has received considerable attention, signaling pathways utilized within other subcellular organelles have been less extensively explored. A growing body of evidence now indicates that the endoplasmic reticulum (ER) plays a central role in cell signaling and mediates many neurodegenerative processes. Disturbances in the ER triggers stress signaling by two distinct pathways, the unfolded protein response (UPR) and the overload response (EOR) (for review see (3). The UPR components were first identified in yeast (see for review (4) and, to date, three signaling proteins have been identified in mammalian cells; these are PERK (PKR-like ER kinase), Ire1 (high inositol-requiring) α and β, and ATF-6 (activating transcription factor) α and β. It has not yet been determined what distinguishes the EOR from the UPR, but the signaling that mediates the EOR and UPR are different. The EOR leads to activation of NF-κB and the UPR leads to activation of transcription factors which, in turn, activate ER stress responsive genes, such as grp 78(also called Bip) and grp 94 (see for review (5). These various mechanisms are activated in order to deal efficiently and appropriately with encountered stress. However, severe or prolonged ER stress leads to cell death. The signaling by which the three sensors of the UPR, PERK, Ire 1 and ATF-6 lead to cell death is yet to be elucidated. All of the three sensors (i.e. PERK, Ire1 and ATF-6) converge to activate gadd 153 (also called CHOP), a transcription factor that has been implicated in the induction of apoptosis (for review see (5), by mechanisms that still remain to be determined. One possible explanation is that the ER stress response cascade activates JNK kinase; such activation plays a critical role in regulating apoptosis. Of interest in this regard is that CEP-1347/KT-7515, a JNK inhibitor, has been shown to inhibit cell loss in MPTP-treated mice (6).

In the present study, we have examined whether MPP^+ is toxic to the substantia nigra in the New Zealand white rabbit. We have administered MPP^+ directly into the cerebrospinal fluid using an injection into the cisterna magnum, and have focused our studies on assessing the effects of this treatment on the ER stress response. Our results demonstrate that MPP^+ is neurotoxic to rabbit, since it reduces the tyrosine hydroxylase-positive cell population in the substantia nigra. The apparent mechanism for this is that MPP^+ induces an ER stress response involving activation of the transcription factors, ATF-6 and NF-κB, the activation of gadd 153 and the activation of caspase-3; the latter effect correlates with the activation of JNK kinase as well as one of its substrates, c-jun.

MATERIALS AND METHODS

Antibodies

The following antibodies were used for immunohistochemistry and Western blot analyses: mouse monoclonal antibodies (mAb) to tyrosine hydroxylase (Chemicon, Temecula, CA), ATF-6 and caspase-3 (Active Motif, Carlsbad, CA), goat anti-PERK and Ire1 polyclonal antibodies, and mAbs recognizing gadd 153, NF-κB, JNK and p- JNK, p c-jun and histone H1 (Santa Cruz Laboratories, Santa Cruz, CA), calnexin (Transduction Laboratories, Lexington, MD), mAbs to cytochrome c oxidase subunit IV and KDEL (detects both grp 94 and grp 78) (Stressgen, San Diego, CA).

Animals and treatment

Adult (2 years and 4-5 kg) female New Zealand white rabbits received intracisternal injections of 100 µL of normal saline (n=6), 100 µL of 4 mM (400 nmol) MPP$^+$ iodide (Sigma Chemical Co., St Louis, MO) in saline (n=6), or 100 µl of 10 mM (1 µmol) MPP$^+$ iodide in saline (n=6). The injections were carried out slowly over a period of 2 min under ketamine anesthesia. The animals were sacrificed at day 7. At sacrifice, rabbits were perfused at 37°C with Dulbecco's phosphate-buffered saline and the brains were promptly removed and cut to yield two symmetrical hemispheres, one for immunohistochemistry and the other for Western blot analysis. The respective sides chosen for these studies were alternated between successive animals. Each brain hemisphere intended for tissue sectioning was immediately frozen on a liquid nitrogencooled surface, placed into a zipper-closure plastic bag, and buried in dry ice pellets until transferring to –80°C for storage before sectioning and immunohistochemistry. For immunoblot analyses, the substantia nigra was isolated by a high cut through the most rostral midbrain in order to separate the brainstem from the cerebrum. The medial inferior portion of the midbrain was dissected out for homogenization. This area corresponds to the substantia nigral region containing the higher density of tyrosine hydroxylase-positive cells and processes.

All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Animal Care and Use Committee.

Immunohistochemistry

Tyrosine hydroxylase and p-c jun.

Coronal frozen sections (14 μm-thick) at the substantia nigra level from control and MPP^+-treated rabbits were air-dried, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of 1.5% normal horse serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse mAb against tyrosine hydroxylase at a 1:1000 dilution or with phosphorylated c-jun at a 1:250 dilution. After washing with PBS and incubating with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin-biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining. For negative controls, using similar sections, normal saline was substituted for the mAb. All procedures were performed at room temperature unless otherwise noted.

Oxidative DNA damage.

We used the OxyDNA assay kit (Calbiochem, San Diego, CA), which is based upon the direct binding of a mAb, fluorescently labeled, to 8-oxoguanine moities in the DNA of fixed tissue. Frozen sections (14 µm thick) at the substantia nigra level from control and MPP^+-treated animals were air-dried, fixed in ice-cold 4% paraformaldehyde in PBS for 15 min, briefly washed in TBS/Tween 20 and permeabilized in ice-cold 99% methanol for 30 min. After 2 washes, the sections were incubated with a blocking solution (provided in the kit) for 30 min at 37°C for 1 hr, washed twice in TBS/Tween 20, incubated overnight at 4°C with a 1:50 dilution of the FITC-conjugate concentrate that contains the binding protein conjugated to fluorescein, washed 5 times in TBS/Tween 20, and counterstained in the dark for 15 min with 1 µg/ml propidium iodide (Sigma). After a 5 min wash in dH_2O, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a fluorescent microscope (Olympus BH2, Melville, NY) using excitation/emission wavelengths of 365/490 nm.

Western blot analysis

Proteins from whole homogenates or from the nuclear, mitochondrial, cytosolic and microsomal fractions, extracted as we have described previously(9, 10), were used when indicated. In brief, substantia nigra-enriched tissue was gently homogenized using a teflon homogenizer (Thomas Scientific, Philadelphia PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/mL pepstatin and 12.5 mg/mL of N-acetyl-Leu-Leu-Norleu-Al). The nuclear fractions were first isolated by centrifuging the homogenates at 750 g for 10 min at 4°C. The mitochondrial fractions were then isolated from the soluble (cytosolic) fraction at 8000 g for 20 min at 4°C. The 8000 g pellets were resuspended in cold buffer without sucrose and used as the mitochondrial fraction. The supernatant was further centrifuged at 100,000 g for 60 min at 4°C to separate the cytosolic from the microsomal fractions. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Illinois). Proteins (5 μg) from the nuclear, mitochondrial, cytosolic and microsomal fractions were separated by SDS-PAGE (10 % gel), followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and incubation with antibodies recognizing ATF 6, PERK, Ire1 and KDEL at a 1:100 dilution and gadd 153, NF-κB, caspase-3, JNK and p- JNK at 1:250. Calnexin mAb was applied as an ER marker at 1:500, histone H1 mAb as a nuclear marker at

1:500, and cytochrome c oxidase subunit IV mAb as a mitochondrial marker at a 1:1000 dilution. A mAb reacting with ß actin (Sigma, Saint Louis, MI) was applied at a 1:250 dilution as a gel loading control. The blots were developed using an enhanced chemiluminescence detection kit (Bio-Rad, Hercules, CA).

RESULTS

Clinical symptoms.

The 1 μmol MPP^+ produces neurological symptoms, particularly gait difficulties and a partial loss of the righting reflex, and it was necessary to sacrifice the animals on day 7.

MPP^+ reduces tyrosine hydroxylase (TH)-positive neurons and induces oxidative DNA damage.

Intracisternal administration of 1 μmol (Figure1a, panel C), but not 400 nmol MPP^+ (Figure 1a, panel B), reduces TH immunoreactivity in the lateral substantia nigra (*pars compacta*) of New Zealand white rabbits in comparison to control (Figure 1a, panel A). While sections from a control (Figure 1b, panel A) or from a 400 nmol MPP^+-treated rabbit (Figure 1b, panel B) do not exhibit 8-oxoguanine immunoreactivity, sections from a 1 μmol MPP^+-treated animal show intense immunoreactivity to this antibody (Figure 1b, panel C). Propidium iodide (P.I) is applied as a nuclear marker (Figure 1b, panels D-

F), and by merging the images it can be demonstrated (Figure 1b, panel I) that 8-oxoguanine immunoreactivity is predominantly localized in the nucleus (arrowheads) and to a lesser extent outside of the nucleus, probably in mitochondria (arrow, small round foci).

MPP$^+$ activates the UPR and the EOR pathways.

While the ATF-6 pathway is activated by MPP$^+$ treatment, as indicated by the Western blot analysis with ATF-6 mAb (Figure 2a), there is no apparent activation of the PERK and Ire1 pathways (data not shown). ATF-6 (90 kDa) is a constitutively synthesized ER protein and our result show that ATF-6 is exclusively localized in the ER in controls. In the 400 nmol MPP$^+$-treated animals, the 90 kDa band is detectable in the ER and the cytosolic fractions. In the 1 μmol MPP$^+$-treated rabbits, ATF-6 is present as the 90 kDa band in the cytosol and as two intense bands (60 kDa and 50 kDa) in the nuclear fraction (Figure 2a).

The inducible transcription factor, NF-κB, is normally sequestered in the cytoplasm by the inhibitory unit IκB, and translocates to the nucleus following cytotoxic insults. Our results show that NF-κB is detected as a 100 kDa band in the cytosolic fraction from the controls, 400 nmol MPP$^+$-treated and 1 μmol MPP$^+$-treated animals; two additional bands of 100 and 65 kDa are detected in the nuclear fraction of the 1 μmol–treated animals (Figure 2b).

The purity of the different subcellular fractions used for these studies is shown to be satisfactory, as indicated by the subcellular markers (Figure 2c); cytochrome c oxidase subunit IV (Cyt.ox) stains mainly the mitochondrial fraction, calnexin stains the ER fraction and histone H1 the nuclear fraction.

MPP+ induces translocation of grp 94, grp 78 and gadd 153 to the nucleus.

In controls, the ER-resident proteins, grp 78 and grp 94, are detected in the ER fraction (Figure 3a). In the 400 nmol MPP$^+$-treated rabbits, grp 78 and grp 94 are primarily localized in the nuclear fraction. In the 1 μmol MPP$^+$ -treated rabbits, the grp 78 and grp 94 bands are less intense in the nuclear fraction than in the cytosolic (both bands) or ER fractions (grp 78) (Figure 3a and b). The grp 94 band is absent in the ER fraction (Figure 3b).

The protein gadd 153 is a non-ER-resident protein that is present in the cytosolic fractions in controls and in the 400 nmol MPP$^+$-treated animals; following administration of 1 μmol MPP$^+$, gadd 153 is detected in both the cytosolic and the nuclear fractions (Figure 3b).

MPP$^+$-induced ER stress correlates with the cleavage of caspase-3 and activation of JNK kinase.

Western blot analysis demonstrates the presence of a pro-caspase-3 band (32 kDa) in control and in MPP+-treated animals; caspase-3 p20 and p17, two activated forms of caspase-3, while not detectable in control or in 400 nmol MPP^+-treated animals, are present in the 1 μmol MPP^+-treated rabbits (Figure 4a).

The mAb we used in these studies to detect JNK does not differentiate between JNK1 and JNK2. JNK is detected as a double band of 54 and 46 kDa in controls and in the 400 nmol and 1 μmol MPP^+-treated animals (Figure 4b). Also, the mAb employed for detecting active JNK (p-JNK) does not differentiate between phosphorylated JNK1 and JNK2. The active p-JNK is only slightly detectable as a double band in control and in the 400 nmol MPP^+-treated animals. Administration of 1 μmol MPP^+ dramatically increases the levels of p-JNK (Figure 4c).

MPP^+-induces the phosphorylation of c-jun.

Phosphorylated c-jun staining was examined in sections at the level of the substantia nigra (Figure 5, box in panel A). In sections from controls, cells are not stained with the phospho c-jun mAb (Figure 5, panel B). The 400 nmol MPP^+ induces very few positive-scattered cells in the substantia nigra (Figure 5, panel C), whereas 1 μmol MPP^+ produces a marked phospho c-jun immunoreactivity of cells (Figure 5, panel D).

DISCUSSION.

Administration of MPP^+ via the intracisternal route produces a dose-dependent loss of tyrosine hydroxylase immunostaining and oxidative DNA damage in the substantia nigra of New Zealand white rabbits. This treatment also induces activation of the two ER stress response pathways, UPR and EOR, as indicated by the translocation into the nucleus of gadd 153 and NF-κB respectively. The neurotoxic effect of MPP^+ correlates with cleavage of the effector of apoptosis, caspase-3, and the activation of the MAP kinase family member JNK, as well as activation of the transcription factor c-jun.

We have employed New Zealand white rabbits for our *in vivo* experiments as an alternative to the more commonly used rodent *in vivo* and *in vitro* systems, or to the less available primate. Rabbits may be a valuable resource for the study of neurotoxic agents since genetically they more closely resemble primates than rodents (11). We also have shown that this rabbit species is very susceptible to neurotoxin-induced neurodegeneration (12). Previous work has demonstrated differences in the susceptibility of different strains of rabbit to MPTP neurotoxicity (13). In the latter study, the intraperitoneal injection of MPTP causes a 40% reduction in striatal dopamine content in "little-silver-black " and no change in "chinchilla" rabbits. This difference in MPTP toxicity probably represents a difference in MPTP metabolism in these breeds. In our animal model system we have overcome these problems by injecting MPP^+ directly into the cerebrospinal fluid of the brain via the cisterna magnum and, as shown in our results, this mode of administration in New Zealand white rabbits is neurotoxic to dopaminergic neurons in the substantia nigra.

A growing body of evidence now indicates that the endoplasmic reticulum plays a central role in cell signaling and that it mediates many neurodegenerative processes. This intracellular organelle is of fundamental importance, since it serves as a dynamic Ca^{2+} store, with a high free Ca^{2+} concentration maintained within its lumen. Besides being a Ca^{2+} storage site, the ER lumen is the cellular compartment where surface and secreted proteins are synthesized, folded and assembled before being transported. Alterations in Ca^{2+} homeostasis lead to a disturbance in correct protein folding and result in the accumulation of misfolded proteins within the ER. Disturbances in Ca^{2+} homeostasis and protein processing trigger stress signaling from the ER by two distinct pathways, the UPR and the EOR (for review see (3). This signaling is intended to provide cells with the capability to deal with changes in Ca^{2+} levels and with the toxic buildup of misfolded proteins. Although ATF-6, Ire-1 and PERK share the same protein and transcription factor targets, the relationship between the three proteins remains to be clarified. Furthermore, concomitant activation of the three signaling proteins is not required; activation of only one of the three is sufficient to activate the UPR cascade.

ATF-6 is a constitutively synthesized ER protein that binds directly to the cis-acting ER stress-response element (ERSE) responsible for the mammalian UPR (14,15). Upon ER stress, ATF-6 is first transported to the Golgi where it is cleaved in the nucleus and activates the ER-resident chaperones, grp 94 and grp78, and gadd 153 (16, 17). However, up to the present time, not all the ATF-6-target genes have been fully identified. Our results show that 1 μmol MPP^+ clearly activates ATF-6, leading to cleavage in the nucleus. We also demonstrate that cleavage of ATF-6 correlates with activation of the target proteins, grp 94 and grp 78.

Increased levels of NF-κB activity have been observed in the brain of patients with various neurodegenerative disorders including Parkinson's disease (18). NF-κB, is an inducible transcription factor and is an important mediator of the human immune and inflammatory response. Exposure of cells to various pathological stimuli activates NF-κB; activated NF-κB dimer is rapidly released from the cytoplasm, where it is normally sequestered by the inhibitory unit IκB, and then translocates to the nucleus. Various agents that induce stress in the ER have been shown to activate NF-κB (19-21). The role of NF-κB in regulating neuronal death is complex. In some cases it has been demonstrated to promote neuronal survival, and in other cases to induce neuronal death. It appears that whether NF-κB acts as a promoter or inhibitor of neuronal loss depends on the cell type and the nature of the toxic stimuli. Our data show that the higher, but not the lower MPP^+ concentration induces activation of NFκ-B, and that this activation correlates with the activation of caspase-3. Caspase-3 is an effector of apoptosis and may represent the last step in the initiation of apoptosis prior to DNA fragmentation.

Grp94 and grp78 are ER-resident proteins that are activated in response to ER stress and serve as molecular chaperones. These proteins are involved in protein translocation, protein folding and assembly, and the regulation of protein secretion. Their expression is induced during cytotoxic stimuli including oxidative stress, chemical toxicity, treatment with Ca^{2+} ionophores and inhibitors of glycosylation (3). Activation of these chaperones during the UPR is necessary to alleviate ER stress, maintain ER function, facilitate protein folding and thus protect cells from toxic insults. However, mechanisms that underlie the cellular protection of these ER stress-chaperones are still unknown.

Activation of gadd 153 expression has been demonstrated following transient cerebral ischemia in the rat (22). It is a non-ER-resident protein that is specifically activated in response to stress in the ER. Upon activation, gadd 153 translocates from the cytoplasm to the nucleus where it may participate in induction of apoptosis. The gadd 153 gene is specifically activated by agents that disturb ER function such thapsigargin, an agent that perturbs intracellular free calcium. mRNA levels for gadd 153 has been demonstrated to be increased both during hypoxia and after exposure of cells to agents that elevate the levels of glucose-regulated proteins (23). The exact mechanism by which gadd 153 kills or participates in cellular death remains to be elucidated. Our results demonstrate that the non-toxic effect of the 400 nmol MPP^+ correlates with increased levels of grp 94 and grp 78 and with no gadd 153 in the nucleus. The marked translocation of gadd 153 to the nucleus, we show with the neurotoxic 1 μmol MPP^+, is accompanied by a decrease in the level of grp 94 and grp 78 proteins in the nucleus.

Caspase-3 is an effector caspase and its activation may occur through different pathways. This activation can result from cytochrome *c* release by the mitochondria and activation of caspase-9, which in turn activates caspase-3. Caspase-3 can also be activated by caspase 8 through death receptors. Perturbation of ER function and release of caspase-12 can also activate caspase-3 (24). The activation of caspase-3 that we observe with treatment with 1 μM MPP^+ may result from activation of one or more of these three different pathways.

Recently, evidence that ER stress contributes to neuronal death has been demonstrated in an vitro model of Parkinson's disease where incubation of PC12 cells with MPP^+ induces the phosphorylation of the endoplasmic reticulum kinases Ire1 α and PERK (8). Treatment of SH-SY5Y cells with MPP^+ has been demonstrated to increase the expression of gadd 153; rotenone or 6-hydroxydopamine, whose modes of action are via mitochondrial impairment and oxidative stress respectively, did not exhibit this effect (7). These latter results as well as our results suggest that MPP^+ targets the ER directly.

Evidence has implicated the JNK kinase in the death of dopaminergic nigral neurons in the MPTP model of Parkinson's disease *in vitro* (25) and in *in vivo* (26). In nigral cultures, MPP^+ treatment induces JNK activation, an effect that precedes the maximal induction of apoptosis (27). MPP^+ also activates JNK and NF-κB in SH-SY5Y neuroblastoma cells (28). However, in these studies no attempt was made to determine the possibility that ER stress is the inducer of JNK.

In the present report we find that MPP^+-induced toxicity correlates with phosphorylation of c-jun in the substantia nigra. JNK activities are regulated in the cytoplasm by phosphorylation of threonine (Thr^{183} for JNK1 and JNK2) and tyrosine (Tyr^{185} for JNK1 and JNK2) residues. Some of the physiological and apoptotic actions of the JNKs include the phosphorylation of c-jun (see for review (29).

Collectively, our results demonstrate that MPP^+ is neurotoxic to rabbit brain by mechanisms that involve stress in the ER and activation of the JNK kinase signaling pathway. Prevention of ER stress or the inhibition of JNK kinase activity may thus be necessary to prevent MPP^+ toxicity and may represent a new therapeutic approach in the prevention of neurotoxin-induced Parkinson's disease.

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APPENDIX VI

MPP+ INDUCES THE ENDOPLASMIC RETICULUM STRESS-RESPONSE IN RABBIT BRAIN INVOLVING THE ACTIVATION OF ATF-6 AND NF-κB SIGNALING PATHWAYS

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Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie the MPTP/MPP+-induced neurotoxicity, which has been used extensively as a model for Parkinson's disease. In the present study we examined the hypothesis that MPP+ targets the endoplasmic reticulum. Since rabbits are more genetically similar to primates than to rodents we selected this animal model system for our MPP+ neurotoxicity studies. MPP+ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. MPP+, in a dose-dependent manner, induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by activation of ATF-6 and NF-κB, leads to activation of gadd 153. These effects correlate with the activation of caspase-3 and of JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

Support Contributed By: US Department of the Army and the Environmental Protection Agency

Close

APPENDIX VII

Cellular mechanisms of neurodegeneration and Alzheimer's disease

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of extracellular neuritic plaques (NPs), the formation of neurofibrillary tangles (NFTs), and massive neuronal and synaptic loss. Aβ is the major component of the NPs, with hyperphosphorylated tau being the primary constituent of NFTs; both of these aggregates are generally accepted as being key players in the pathogenesis of AD. Whether Aβ or tau actually represents the primary cause of neuronal loss in AD, and which of the two abnormal changes is generated first and is presumably the more important, remains a matter of debate. Furthermore, the mechanisms by which Aβ and/or tau kill neurons in Alzheimer's disease (AD) are not fully understood. Oxidative stress has been proposed as a major factor in neuronal injury an as an inducer of apoptosis. However, whether apoptosis or necrosis is the major route of cell death remains to be clarified, although an apoptotic mechanism is gaining increasing support.

We have developed a unique animal model where the intracisternal administration of aggregated Aβ(1-42) peptide into rabbit brain induces neuronal death and hyperphosphorylation of tau protein. Death of neurons is mediated by endoplasmic reticulum (ER)-specific apoptosis as is evident by the activation of caspase-12, a specific marker for ER stress, and by activation of the pivotal inducer of apoptosis, caspase-3, which we show to be primarily localized in the ER. These results implicate Aβ as the trigger of tau phosphorylation with the ER is a direct target for Aβ-induced neurotoxicity. We further demonstrate that neurotoxic effect of Aβ correlates with the activation of the JNK and ERK MAP kinases along with the activation of the transcription factors, c-jun and c-fos. Treatment of the experimental animals with lithium or glial cell-derived nerve growth factor (GDNF) prevents induction of ER stress but not hyperphosphorylation of tau. These results suggest that two distinct pathways are involved in the apoptosis cascade activation and in tau phosphorylation following Aβ administration. While the effects of lithium and GDNF on caspase-3 and tau are similar, they differentially regulate the MAP kinases, JNK and ERK pathways. The results of these experiments add extended insight into intracellular molecular signaling pathways that could form the basis underlying neurodegeneration particularly resulting from the neurotoxic effect of Aβ.

APPENDIX VIII

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Neurobiology of Aging 23 (2002) 1089–1092

NEUROBIOLOGY OF AGING

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Commentary

Is amyloid β-peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions?

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Received 23 January 2002; accepted 23 January 2002

Alzheimer's disease is a progressive neurodegenerative disorder with three characteristic neuropathological features, namely the extracellular deposition of amyloid β-peptide (Aβ) [28], the formation of neurofibrillary tangles (of which microtubule-associated protein tau (tau) is a primary component), and a selective loss of neurons. How these features interrelate and which, if any, is of primary importance is still unclear. Selective neuronal death involves vulnerable brain regions, in particular the hippocampus and cerebral cortex, and apoptosis may play a role in the process of cell loss (for review, see [15]).

Robinson and Bishop in their clearly presented and provocative article "Aβ as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease" [24], propose that Aβ accumulation is a neuroprotective response to an upstream adverse event that non-specifically leads to Alzheimer's disease and perhaps other types of neurodegeneration. The authors have developed their hypothesis on an extensive literature that fails to show that Aβ accumulation consistently correlates with neuronal loss and cognitive decline. Transgenic mice which accumulate large loads of Aβ-containing plaques show either no change or only a mild neurotoxic response to these loads. This endogenously produced Aβ may represent the optimal model system for studying aggregated Aβ neurotoxicity. However, as Robinson and Bishop state, "mice are not humans" and they could have a much different response to the presence of a neurotoxin. The other mode for creating a model for an increased burden of aggregated Aβ is to inject this material directly into the brain of experimental animals. As reviewed by Robinson and Bishop, the results of such experiments are equivocal but Aβ injections have been shown to produce neurotoxicity in aged monkeys [10]; however, such treatment of rats does not appear to induce neurotoxicity [2]. Recent studies in our laboratory have employed rabbits as the experimental animal. Rabbits may be particularly relevant to the investigation of human disease since they belong to the mammalian order Lagomorpha, a group reported to more closely resemble primates than rodents [14]. The experiments performed in our laboratory involved the intracisternal injection of aggregated Aβ(1–42) into aged New Zealand white rabbits [12]. Although evidence of severe neurotoxicity was not observed, there was stress to the ER as indicated by reduced levels of the antiapoptotic protein, Bcl-2, in this organelle. Additional stress was suggested by the induction of nuclear translocation of *gadd 153* and the inducible transcription factor, NF-κB. It could be expected that any neuropathological response to this exogenous mode of administration of Aβ might be different from that induced by the Aβ produced endogenously, as is the case in transgenic mice. Exogenous administration certainly has more potential to introduce environmental contaminants such as toxic metals.

The neurotoxic effect of metal ions in transgenic mice carrying an increased burden of Aβ plaques has not been extensively studied. The hypothesis of Robinson and Bishop introduces the possibility that flocculant Aβ deposits possess neuroprotective properties by binding toxic materials, particularly toxic metals, rendering them inactive until they can be eliminated from the central nervous system. In regard to this latter hypothesis, another aspect of metal–Aβ interactions has focused on the possible intimate involvement of copper (Cu), zinc (Zn) and iron (Fe) in Aβ formation and/or biological activity. Aβ(1–42) binds Cu(II) with extremely high affinity [1] and Cu(II), Zn(II) and Fe(III) can induce aggregation of Aβ [3]. Furthermore, brain tissue levels of these metals are elevated in the neocortical region of patients with Alzheimer's disease, and are present in amyloid plaque deposits [19]. These findings can support the hypothesis that

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PII: S0197-4580(02)00037-4

metal-induced aggregation of Aβ is an important step in the pathogenesis of Alzheimer's disease. However, in diseases where free Cu(II) or Fe(III) is available due to an inherited defect related to transport and cellular uptake of these metal species, there is no evidence of an increased formation of Aβ plaques. An example of this is in Wilson's disease, an inherited disorder of copper metabolism, in which there is increased free Cu(II) and considerable evidence of neurotoxicity, but not an increased formation of plaques [9]. Similarly, in neurodegeneration with brain iron accumulation-1 (formerly termed Hallervorden–Spatz disease) there are extracellular proteinaceus accumulations (spheroids), but again no evidence of excessive plaque formation [8]. Although Zn(II), Cu(II) and Fe(III) induce rapid aggregation of Aβ in vitro [3], and agents that specifically chelate Cu(II) and Zn(II) will solubilize Aβ in postmortem brain tissue obtained from Alzheimer's disease patients [7], there is no direct evidence that these metal ions are involved in the Aβ aggregation process in Alzheimer's disease. A recent report has shown that oral treatment of mice transgenic to APP 2567 with clioquinol, stated to be a Cu/Zn chelator, inhibits Aβ accumulation and significantly improves general health and body weight parameters in comparison to sham-treated animals [6]. Clioquinol is a derivative of 8-hydroxy quinoline and is not a specific chelator of Cu(II) and Zn(II), although there is some implication that this is the case by the authors of that article. In this regard, the order of stability constants for binding of 8-hydroxyquinoline derivatives is Fe(III) > Cu(II) ≫ Ni(II) > Al(III) > Zn(II) [21] and the affinity of clioquinol binding to Cu(II) and Zn(II) is much less ($K_1(\mathrm{Cu}) = 8.9$; $K_1(\mathrm{Zn}) = 7.0$) than the attomolar levels reported by Atwood et al. [1].

Robinson and Bishop review other features of Aβ aggregation that are important to the present commentary. *First*: patients with traumatic head injury can rapidly form Aβ plaques within a few days of the injury, but these lesions may eventually disappear [20]. *Second*: Aβ is constantly being produced in the healthy brain. Both of these events suggest that there has to be an additional component present to induce aggregation of Aβ and to render it relatively stable. If this other component is not Cu and Fe, as indicated by the lack of increased plaques in human diseases where these metals accumulate in the brain, then other metals or compounds must be considered. Zinc is still a possibility but this metal is ubiquitous and possesses many vital biological properties. It is unlikely, in our opinion, that Zn would suddenly assume a toxic role in the cerebral cortex of aged brains.

Robinson and Bishop mention aluminum (Al) as a possible toxic metal along with Cu, Zn and Fe. The hypothesis that aluminum is involved in the pathogenesis of Alzheimer's disease is anathema to most neuroscientists, probably due to conflicting data in the literature reflecting the complex chemistry and ubiquitous nature of this element. Aluminum is the most common metal in the earth's crust and is highly toxic at picomolar concentrations. As with Cu and Fe, there is a human disorder that involves the accumulation of Al in the brain. This disease may result from long-term hemodialysis treatment and was quite common 20 years ago when Al-contaminated water was used to prepare dialysate solutions. In this iatrogenic disease, a fatal encephalopathy often developed, but was not associated with Alzheimer's-like pathology except in rare reports [4]. However, the renal-impaired patient is known to accumulate a plethora of abnormal metabolites, including conjugates of phenol, organic acids of the tricarboxylic acid cycle and others that undoubtedly are good Al ligands and would modify its toxic action [29]. Few investigators have attempted to detect Al within neuritic plaques, since the analytical challenges are considerable. Aβ has a molecular weight of ~5000 kDa and since Al(III) has an atomic weight of 27 and is trivalent, it is possible that the ratio of Aβ on a weight to weight basis could be 550:1. Thus a microprobe method for detecting Al within plaques would have to be exquisitively sensitive. Candy et al. [5] reported the presence of Al within plaques in the brain of an Alzheimer's disease patient, although Landsberg et al. [18] failed to confirm this finding with the alternative microanalytical technique of particle-induced X-ray emission (PIXE). However, this nuclear microscopic technique is relatively insensitive below 15 μg/g of Al, making the significance of this report questionable.

Arguments for or against the possible role of Al in neurodegenerative disorders have their strong supporters, but it is quite premature to discard Al as a possible toxic metal that could contribute to the stabilization of Aβ plaques and to their toxicity. The involvement of Al in the plaque hypothesis also provides a possible answer to the formation of the other classical neuropathological feature of Alzheimer's disease, namely the neurofibrillary tangle. It is difficult to conceptualize a mechanism whereby an insoluble mass of proteinaceous material might initiate intracellular events leading eventually to cell death. The fact that such materials, in the case of Aβ, may in one form be non-toxic but as a β-sheet become toxic supports the hypothesis that the injurious agent is bound to the plaque but can be released and gain entry to brain cells. In our laboratory, after the injection of Al maltolate intracisternally into rabbits, the numerous intensely argyrophillic intraneuronal neurofilamentous aggregates which result are immunopositive for hyperphosphorylated tau, abnormally phosphorylated neurofilament proteins, α_1-antichymotrypsin and ubiquitin [16,25], all of which are present in the neurofibrillary tangles of Alzheimer's disease [17]. Interestingly, treatment with an Al chelating agent will cause the bulk of these intraneuronal protein accumulations to disappear [26,27]. Admittedly, the animal model system used in our studies reflects an acute neurotoxicity, and the neurofilamentous aggregates do not demonstrate paired helical filament structures, although the latter may well be a species-related phenomenon as only humans are known to display this structure. However, very few systems have come as close to mimicking the biochemical composition of the Alzheimer's neurofibrillary tangle as has in this Al in vivo model.

One other piece of evidence supporting the hypothesis of the possible role of Al in the Aβ plaque is as follows: Nakagawa et al. have shown, using in vitro experiments with Aβ(1–40), that there is an endoplasmic reticulum-specific apoptosis mediated by a novel caspase, caspase-12 [23]. The endoplasmic reticulum (ER) is a subcellular organelle that plays a key role in protein folding and maintenance of calcium homeostasis. It also contains members of the *Bcl-2* family of proteins such as Bcl-2, Bcl-X_L and Bax which play important roles in controlling neuronal viability and apoptotic death. The role of a perturbed ER function in the pathogenesis of Alzheimer's disease is discussed in a recent review by Mattson et al. where it is proposed that stress to the ER leads to altered proteolytic processing of amyloid precursor protein with a subsequent increase in the vulnerability of neurons to apoptosis [22]. We have shown that Al also induces stress in the ER of rabbit hippocampus as assessed by a decrease in the antiapoptotic and an increase in the proapoptotic proteins, caspase-3 activation and apoptosis [11,13]. As stated above, the administration of Aβ(1–42) via a similar intracisternal route also induces stress in the ER, however, no caspase-3 activation or apoptosis has been observed [12]. It is anticipated that the administration of higher doses of Aβ will induce a more severe neurotoxic response.

In summary, we generally support the hypothesis proposed by Robinson and Bishop that Aβ and its aggregates are non-toxic. As put forward in their excellent commentary, these aggregates (plaques) probably form constantly in the healthy brain but are cleared quickly. In traumatic brain injury, where there is an up-regulation of amyloid precursor protein and an increased production of Aβ, a large number of plaques may be deposited. The formation of the plaques is not dependent on the presence of Cu, Zn or Fe, but could be dependent on other toxic metals such as, but not limited to Al. However, the stabilization of the plaques for long periods of time, perhaps permanently, is dependent on the binding of metals, and again Al is a possibility although this has not been shown experimentally. Since patients with Wilson's disease or with neurodegeneration with brain-iron accumulation-1 do not have increased plaque formation, then Cu and Fe are not likely candidates for the stabilization of plaques, although they could be bound to these aggregates and thus could increase oxidative stress and neuronal injury. Sequestration of essential metals such as Cu, Zn, Fe, selenium and others could also contribute to cell injury. We believe it less likely, as proposed by Robinson and Bishop, that plaque formation is a specific mechanism for chelating toxic metals and perhaps other toxic agents, and in sequestering them for phagocytosis. Rather we favor Aβ aggregation as a physical property of this peptide, a process that may lead to neurotoxic complications if a toxic metal is available to bind and stabilize the aggregate. This process may also bind to other metals, inducing oxidative stress, and may deplete the brain cells of essential trace elements. Aβ aggregation may not be a tightly controlled physiological process; however, amyloid precursor protein itself is probably tightly regulated and Aβ production is largely a byproduct of its metabolism.

The excessive formation of Aβ plaques in transgenic mice overexpressing amyloid precursor protein is likely due to an over-production of Aβ, exceeding the cell's ability to metabolize and clear this peptide. Interesting future experiments could involve exposing these animals to toxic metals to ascertain whether the plaques can be stabilized and whether neurotoxicity ensues. A request for proposal to study the APP/PS1 transgenic mouse exposed to Al maltolate in the drinking water has been issued recently by Health Canada, and the results from such studies should help clarify the possible relationship between Aβ plaques and exposure to a neurotoxic metal.

Acknowledgments

We gratefully acknowledge helpful discussions with Dr. R. Bruce Martin, Emeritus Professor in the Department of Chemistry at the University of Virginia. Supported by Grant # DAMD 17-99-1-9552 from the US Department of the Army and the Virginia Center on Aging.

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APPENDIX IX

Aβ induces hyperphosphorylation of tau and apoptosis in rabbit brain, involving activation of GSK-3β and JNK pathways.

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Alzheimer's disease is characterized by deposition of Aβ, formation of neurofibrillary tangles involving hyperphosphorylation of tau protein, and neuronal loss in vulnerable brain regions. There is evidence that accumulation of Aβ aggregates is an upstream process that leads to tau hyperphosphorylation and accumulation, and eventually neuronal death, although the mechanisms underlying these processes is unclear. We have developed an animal model system to study the effect of Aβ by its direct (intracisternal) injection into rabbit brain. Using this system we have investigated the effect of Aβ on GSK-3β and JNK pathway signaling, activation of which is known to interfere with apoptotic neuronal death cascade and to induce hyperphosphorylation of tau. Adult (2.5-3 years and 4-5 kg) female New Zealand white rabbits received either intracisternal injections of 100 μL normal saline (n=6; controls) or 100 μl of 2 mg/ml Aβ(1-42) (n=6; Aβ-treated group). Rabbits were sacrificed after 7 days and perfused with Dulbecco's phosphate buffered saline. The brains were promptly removed, and a coronal section cut and bissected to yield two symmetrical hippocampal segments, one for Western blot analysis and the other for immunohistochemistry. Our results show that Aβ induces activation of GSK-3β, by its translocation into the nucleus, phosphorylation of JNK, and hyperphosphorylation of tau at Ser 202 as measured by the AT8 mAb. This Aβ treatment triggers cytochrome *c* release from mitochondria to the cytoplasm, decreases the levels of the antiapoptotic Bcl-2 protein, and activates caspase-3 together with the transcription factor NF-κB. Thus, we have demonstrated that Aβ triggers tau hyperphosphorylation and apoptosis by mechanisms involving simultaneous activation of GSK-3β and JNK pathways. We suggest that treatment with agents that prevent activation of GSK-3β and JNK pathways would represent a therapeutic strategy for the prevention of Aβ-induced neurodegeneration.

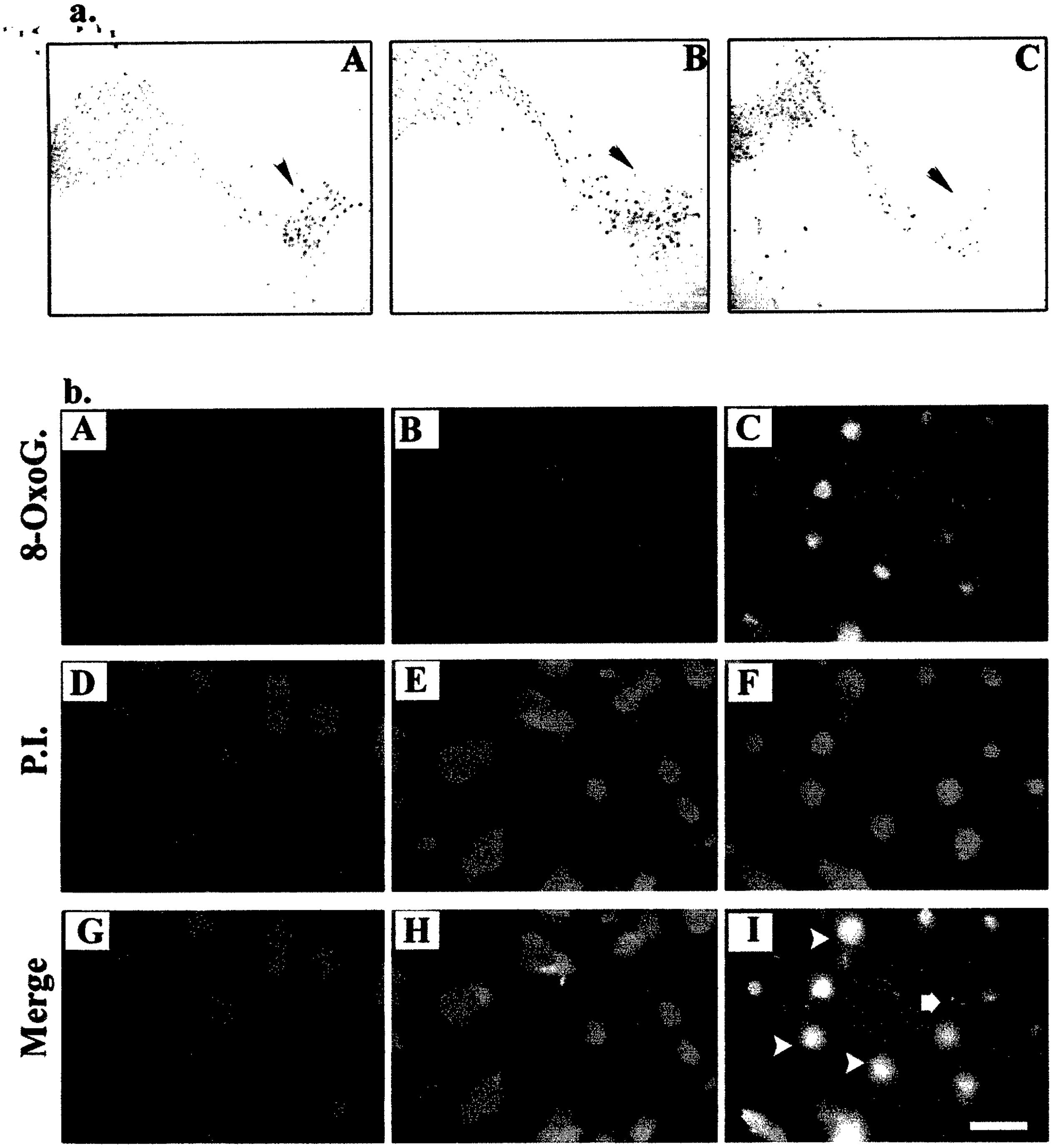

a.
A
B
C
b.
8-OxoG.
P.I.
Merge
A
B
C
D
E
F
G
H
I

Figure 1.

Best Available Copy

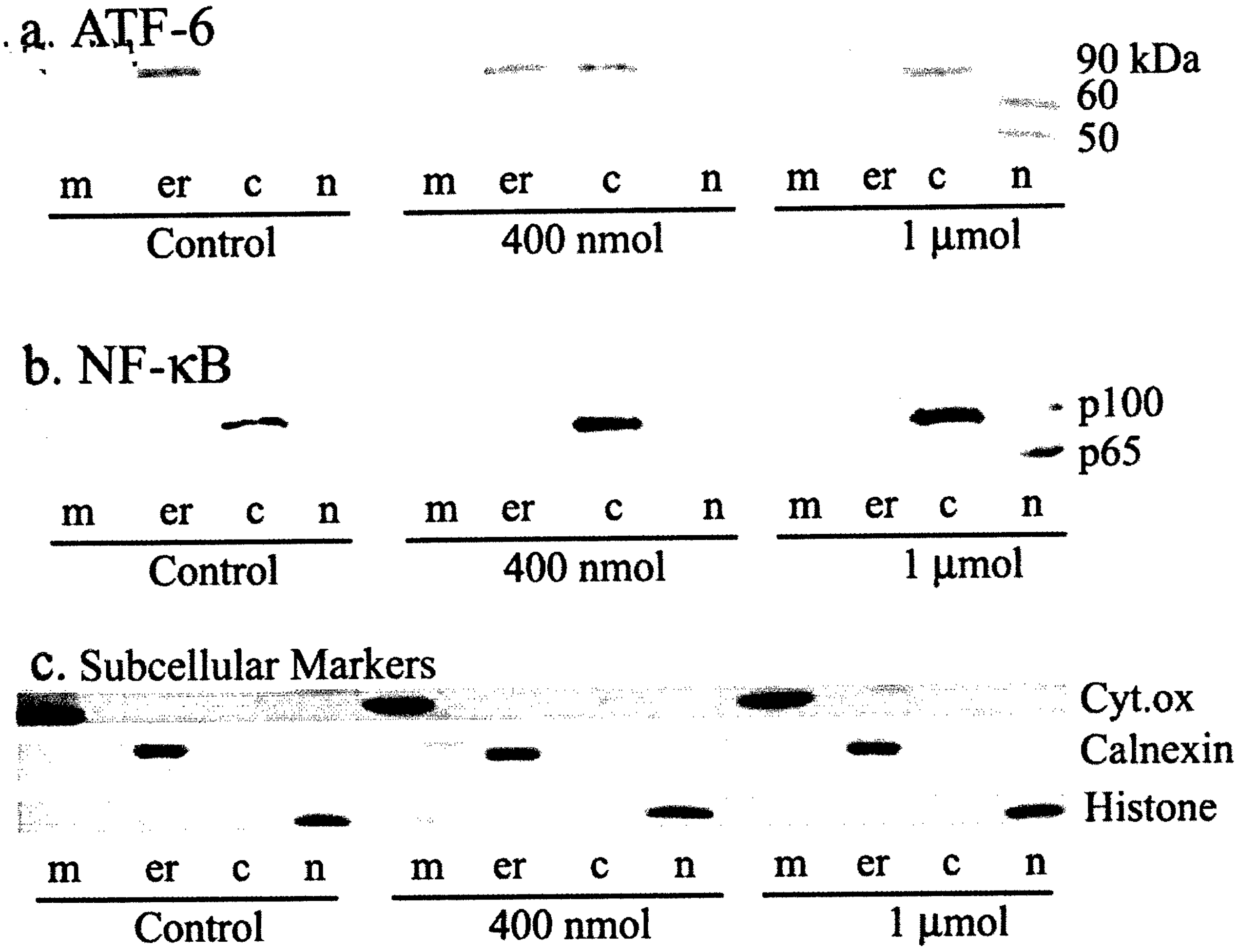
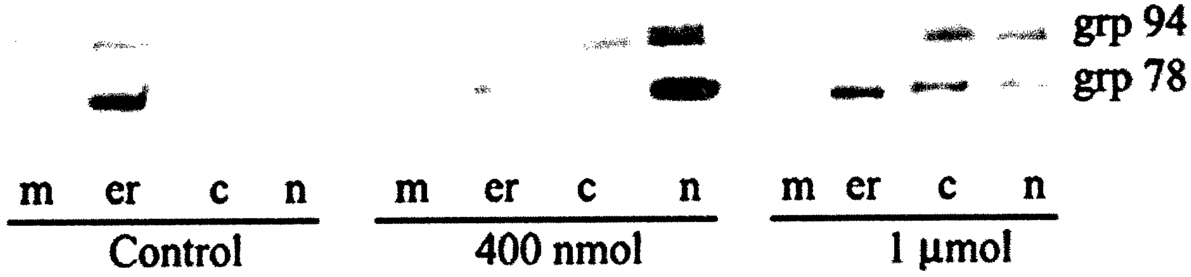

a. ATF-6
90 kDa
60
50
m er c n
Control
m er c n
400 nmol
m er c n
1 µmol
b. NF-κB
p100
p65
m er c n
Control
m er c n
400 nmol
m er c n
1 µmol
c. Subcellular Markers
Cyt.ox
Calnexin
Histone
m er c n
Control
m er c n
400 nmol
m er c n
1 µmol

Figure 2.

a. KDEL


grp 94
grp 78
m er c n
Control
m er c n
400 nmol
m er c n
1 µmol

b. Gadd 153

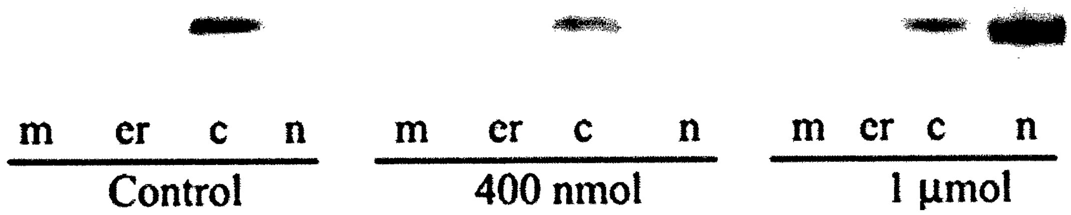
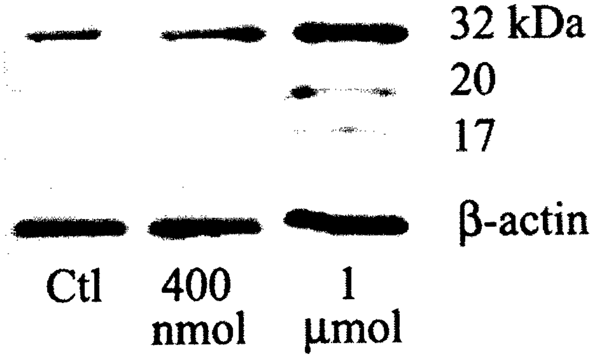
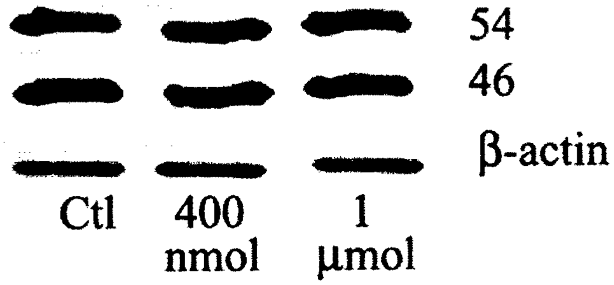

m er c n
Control
m er c n
400 nmol
m er c n
1 µmol

Figure 3.

a. Caspase-3


32 kDa
20
17
β-actin
Ctl
400 nmol
1 µmol

b. JNK


54
46
β-actin
Ctl
400 nmol
1 µmol

c. p-JNK

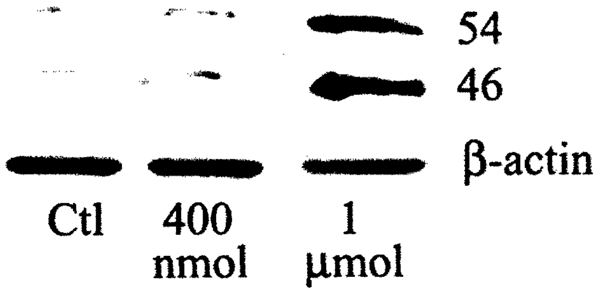

54
46
β-actin
Ctl
400 nmol
1 µmol

Figure 4.

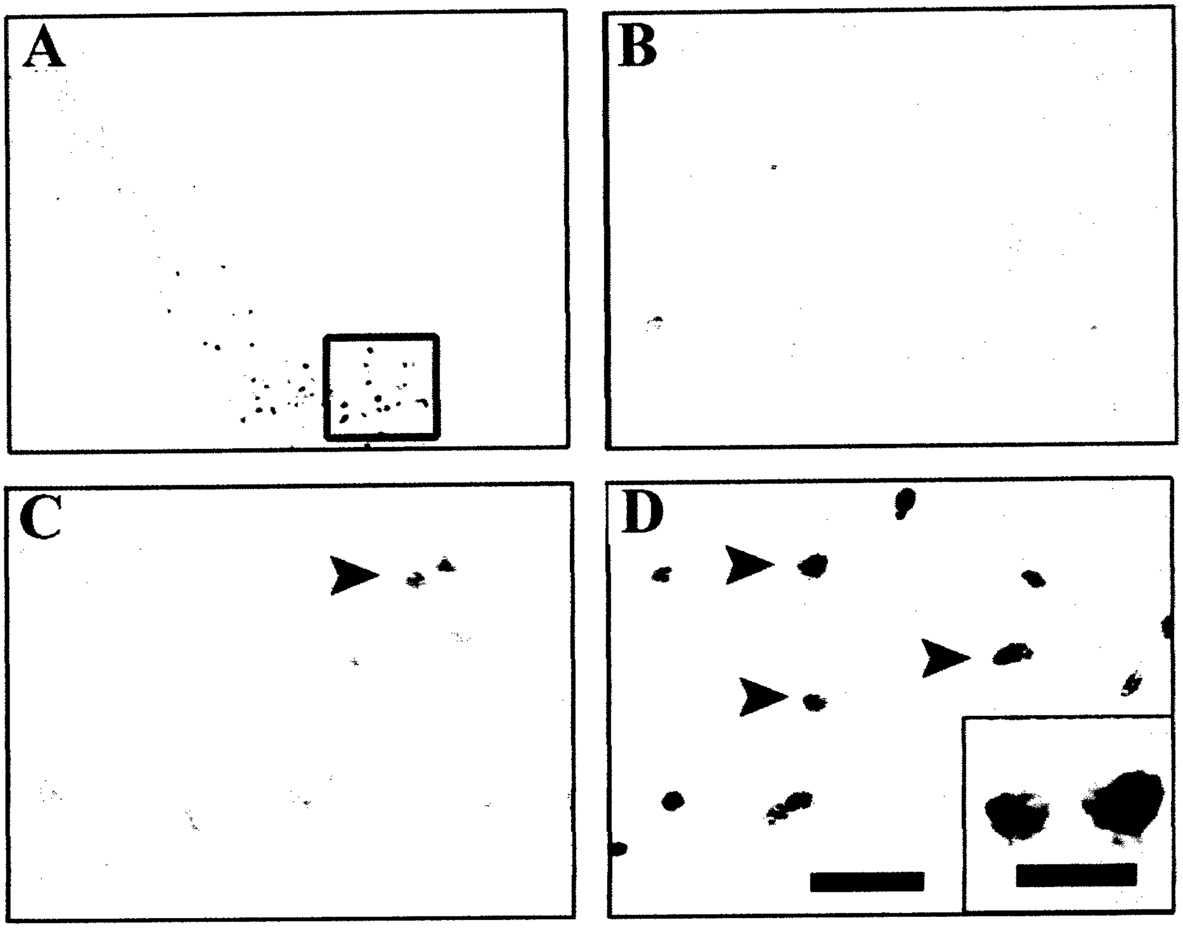


Figure 5.